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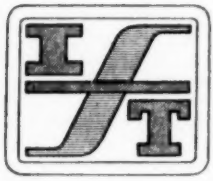

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INSIDE SCIENCE

The Vital Story of VITAMIN C

by Science Writer

What does vitamin C do for human beings?

Medical research shows that it is essential in maintaining healthy bony tissue and for the formation of collagen which is the main supportive protein of skin, tendon, bone, cartilage and connective tissue. It is needed for vascular function and tissue respiration and in promoting the healing of wounds. In lactating women it has great importance in providing the infant with a sufficient amount of ascorbic acid to prevent scurvy, a disease to which very young babies are susceptible.

Much evidence is available in medical reports to show that a lack of this vital nutritive element can result in impaired health. And in those cases of frank scurvy which still occur, ascorbic acid is the specific medication.

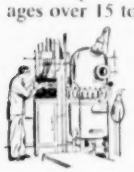
History. The history of vitamin C is fascinating. Scurvy was once the most prevalent disease in Europe. In the 18th Century the antiscorbutic value of fresh vegetables and citrus fruits was recognized.

Isolation. It was not until 1918-25 that almost pure ascorbic acid was concentrated from the lemon by Zilva and its important properties and molecular composition established. In 1927 he concluded that this so-called "reducing factor" and the antiscorbutic principle were closely related. In 1928, Szent-Györgyi, who started investigations in 1921, isolated a strong reducing compound from adrenal glands, oranges, cabbage, terming it "hexuronic acid."

Chemistry. The "reducing factor," "hexuronic acid," and vitamin C were all identified as the same in 1932 by various groups of workers. This was one year after C. G. King established the chemical identification of vitamin C.

Synthesis. In 1933 Reichstein and his colleagues in Switzerland successfully synthesized ascorbic acid. Almost simultaneously in England Haworth and associates accomplished the same. The Reichstein synthesis formed the basis for large scale commercial production by the world-famous firm of Hoffmann-La Roche.

Production. Following these discoveries came the establishment of manufacturing operations so that today daily production by the tons is the rule. The figures are really staggering. The amount produced weekly in the U. S. A. averages over 15 tons, equivalent to that contained in over 200 million oranges. It is remarkable that ascorbic acid can be produced synthetically and sold for only about $21. per kilogram. One kilo is sufficient to supply 92 people for one year with the minimum daily requirements set by the U. S. Food and Drug Administration.



Vitamin C made by the Roche process is identical chemically and in biological activity with Nature's own product. Because vitamins can be manufactured at a much lower cost than if they were extracted from natural sources they are widely used in dietary supplements.

Ascorbic acid is present in many foods. Nature supplies some with lavish amounts and stints on others. In fact, wide variations* have been found in the vitamin C content in varieties of the same fruit.

Deficiencies. Many surveys have shown widespread vitamin C deficiencies in the population. Physicians may call these subclinical, but they are deficiencies nevertheless.

Because of these situations—natural variation and widespread deficiencies—diet experts believe that it is desirable and in the public interest to standardize the vitamin C content of processed fruit and vegetable juices by the addition of enough pure crystalline ascorbic acid to make them consistently dependable sources of this necessary food element. Some of these juices are: tomato, grape, orange, grapefruit, pineapple.

Requirements. The U. S. Food & Drug Administration has set 30 milligrams as the adult minimum daily requirement of ascorbic acid. This daily intake is just about sufficient to prevent recognizable signs of vitamin C deficiencies although a subclinical or an unrecognized deficiency may exist. In view of this, the National Research Council has established 75 milligrams as the recommended daily male adult allowance and 70 mg. daily for female adults, which levels are supported by many controlled nutrition studies. However, the N. R. C. recommends 90 mg. daily for boys between 13 and 15, 100 mg. daily for boys between 16 and 20, and 150 mg. daily for lactating women.

This article is published in the interests of pharmaceutical manufacturers and food processors who make their good products better with essential, health-giving vitamin C. Reprints are freely available. Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada: Hoffmann-La Roche Ltd., 286 St. Paul Street, West; Montreal, Quebec.

*The table shows minimum and maximum levels of ascorbic acid in commercially canned juices. All figures are in milligrams per 100 grams of juice. Data from U. S. Department of Agriculture.

	Min.	*Max.*
Apple juice	0.2	3.6
Grape juice	0.0	4.7
Grapefruit juice	10.0	49.0
Orange juice	9.7	70.0
Pineapple juice	5.4	18.0
Tomato juice	2.5	32.0

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THE CONTROL OF BACTERIA IN CHICKEN SALAD. I. *MICROCOCCUS PYOGENES* VAR. *AUREUS*[a]

H. H. WEISER,[b] A. R. WINTER,[c] AND MARTHA N. LEWIS[d]
The Ohio State University, Columbus, Ohio

(Manuscript received May 2, 1953)

Several hundred food poisoning outbreaks occur annually in this country. Many of them are reported following banquets, church suppers, and other occasions where food is prepared in large quantities and held without adequate refrigeration for several hours before it is used.

Feig *(4)* reported that *Micrococcus pyogenes* var. *aureus (Staphylococcus)* is the principal causative organism of food poisoning. The principal food sources of food poisoning in order of the number of outbreaks are, according to Feig, as follows: meat, poultry products, bakery products, milk products, and vegetables.

Haynes *(7)*, Lumsden *et al. (14)*, Smith and Nagle *(17)*, Lawson and Englar *(11)*, Dauer *(3)*, and others have reported food poisoning outbreaks in hospitals and elsewhere, traceable to micrococcal bacteria in diced chicken and chicken salad not properly refrigerated. The boning and dicing or grinding of chicken after cooking and cooling provide opportunities for contamination from the hands of food handlers *(6)*.

The objectives of this study were to determine the influence of holding temperature, time, and salad ingredients on the growth of micrococcal and standard plate count bacteria in chicken salad.

EXPERIMENTAL

Chicken salad was prepared in quantity food service amounts according to the formula and procedure of Fowler and West *(5)*. It consisted of 5 pounds of cooked and chopped chicken meat, 3 quarts of diced celery, ¼ cup salt, 2½ cups salad dressing, and one cup pickle relish. Commercial salad dressing and pickle relish were used. The salad dressing contained egg yolk, vegetable oil, cereal, vinegar, sugar, salt, and spices. The pickle relish consisted of chopped cucumbers, sugar, distilled vinegar, peppers, spices, and alum. In some trials two to four times the above amounts of ingredients were used. No special sanitary measures, other than good kitchen practices, were followed in preparing the chicken salad.

Micrococci were added to half of each lot of salad. The other half was held as the uninoculated control. The inoculum consisted of one to 6 strains of *Micrococcus pyogenes* var. *aureus* obtained from food poisoning outbreaks. The strains were grown in tryptose broth for 24 hours at 37°C.(99°F.); diluted 1:100 in physiological saline; and, combined just before use. Ten to 100 ml. of the diluted mixture were added to the salad, a few drops at a time, with thorough mixing in a large bowl.

Bacterial analyses were made according to standard techniques before and after various holding temperature and time conditions. The chicken salad and ingredients were sampled by taking a tablespoon of material from various locations in the container; mixing the material and weighing about a 10-g. sample into a tared blender jar. Nine times its weight of water was added and the mixture blended in a Waring

[a] This study was made possible by a grant-in-aid from the Poultry and Egg National Board to The Ohio State University Institute of Nutrition and Food Technology.

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[c] Department of Poultry Science.

[d] Director, Dietary Department, University Hospital.

blender. The resulting mixture was the one-in-ten dilution. Micrococci counts were made in duplicate, on Bacto phenol red mannitol agar containing 7.5% salt, according to the method of Chapman *(1)*. Standard plate counts were made on Bacto tryptone glucose extract agar. The plates were incubated at 37°C.(99°F.) for 48 hours before the colony counts were made.

Temperature recordings were made with an automatic Leeds and Northrup temperature recorder. The chicken salad temperature was taken with a thermocouple inserted in the center of the mass of salad. Room and refrigerator temperatures were recorded with thermocouples located at the height of the salad containers and about 12 inches from them.

Ingredients used in making the salad were analyzed individually and in various combinations before and after inoculation and before and after various holding conditions, as in the case of the prepared salad.

The pH of the salad and ingredients was determined on a sample from the one-in-ten dilution used for the bacterial analysis.

Containers used, holding temperature, and time. Chicken salad was held in containers, varying in size and shape, since they influence food temperature changes as recently re-emphasized by Lewis *et al. (13)*. Shallow pans (2 in. from surface to center), 10-pound, round bottom, metal bowls (4 in. from surface to center) and 30-pound frozen food cans (5 in. from surface to center) were used. Inoculated and control containers of salad were held continuously at 80°F.(27°C.); alternately at 80°F. (27°C.) for 6 hours and 43°F.(60°C.) for 18 hours; and, continuously at 43°F.(6°C.) for 3 days.

RESULTS AND DISCUSSION

It required about 3 hours for the center of the salad to reach the surrounding room temperature when the pans were transferred from the warm room to the refrigerator and vice versa. There was a lag of about 12 hours before the standard plate and micrococcal bacteria began to grow, when the salad was held in a warm room (Figure 1). The uninoculated control lot of salad contained many bacteria, of which more than 50% were micrococci. This confirms the work of Getting *et al. (6)*, that chicken salad becomes contaminated with micrococci during its preparation. The pH of the lots of

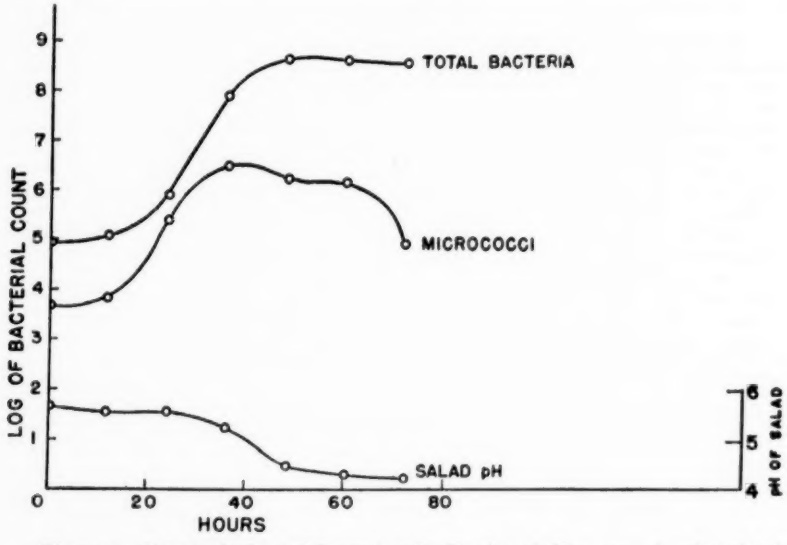

Figure 1. Changes in bacterial count and pH of a shallow pan of uninoculated chicken salad held 72 hours at 77°F.(25°C.) (Trial 4).

salad at time of preparation varied from 5.5 to 5.7. As the bacterial content of the salad increased, the pH decreased.

Ten-pound bowls of chicken salad held alternately at room and refrigerator temperatures (Table 1) showed no appreciable change in bacterial content or pH. This was probably due to the lag period for growth as shown in Figure 1. Also, the salad was not above the temperature required for growth of micrococci more than about 5 hours at a time (Figure 2). Segalove and Dack *(16)*, Jensen *(9)*, and others have reported that the growth of micrococci is insignificant below 48°F.(9°C.).

Thirty-pound cans of chicken salad were held under various conditions (Table 1). When removed from the refrigerator and held at room temperature for 12 hours, there was appreciable increase in bacterial content. As in the preceding trials with smaller containers, the standard plate and micrococcal bacterial counts did not begin to increase rapidly in the large containers until they had been held at room temperature for 12

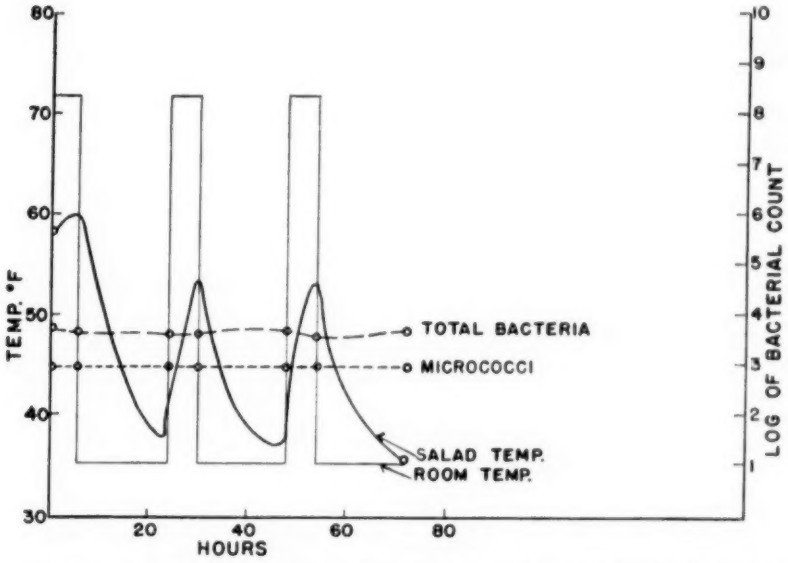

Figure 2. Temperature and bacterial changes in a 10-pound uninoculated bowl of chicken salad held alternately at 73°F.(22.8°C.) for six hours and under refrigeration at 35°F.(1.7°C.) for 18 hours on three successive days (Trial 6).

hours or longer. It required about 24 hours for a 30-pound can of chicken salad to cool from 79°F.(26°C.) to 48°F.(9°C.), the minimum temperature for the growth of micrococci, in a refrigerator operated at 47°F.(8°C.) (Figure 3). It is therefore logical to expect the bacteria to continue to grow for several hours after a big container of chicken salad has been placed in the refrigerator (Figure 3).

Acid studies. The slow growth of micrococci observed in the preceding holding tests may have been influenced by the low pH of the salad and the presence of acetic acid (vinegar) used in some of the recipe components. Hucker and Haynes *(8)* reported that as little as 0.05% acetic acid reduced the growth rate of micrococci. Similar bactericidal effects of acetic acid have been reported by Levine and Fellers *(12)*, Nunheimer and Fabian *(15)*, and Wethington and Fabian *(18)*. The latter investigators also showed micrococci were not able to survive in appreciable numbers for a very long period in mayonnaise and salad dressing of pH 5.6. Kintner and Mangel *(10)* also have reported poor growth of micrococci in salad dressing due to its low pH.

TABLE 1

Influence of holding conditions on the bacterial content of chicken salad (av. of two or more trials)

Holding conditions	Time, hrs.	Inoculated			Control		
		Log. of bact. count			Log. of bact. count		
		Standard			Standard		
		pH	plate	Staph.	pH	plate	Staph.
Shallow pans held at 75°F. to 77°F. (24°C. to 25°C.) for three days.	0	5.6	6.6	6.4	5.7	5.0	3.6
	12	5.7	6.8	6.6	5.6	5.2	3.9
	24	5.7	7.4	7.2	5.7	5.9	5.5
	36	5.3	7.6	7.9	5.5	7.6	6.2
	48	4.6	8.9	7.9	4.6	8.8	6.2
	60	4.5	8.9	7.8	4.3	8.9	6.0
	72	4.2	8.6	7.7	4.2	8.7	4.9
Shallow pans held alternately 6 hours at 80°F.(27°C.) and 18 hours at 43°F.(6°C.) for three days.	0		5.9	5.5		4.5	
	6		6.2	5.7		4.4	
	24		5.4	5.7		2.3	
	30		6.2	6.1		4.8	
	48		6.1	6.0		4.9	
	54		6.4	7.5		5.2	
	72		6.5	6.2		5.5	
Shallow pans held 3 days at 39°F. (4°C.).	0	5.7	6.5	6.3	5.7	5.0	3.6
	72	5.5	6.5	6.3	5.5	5.1	3.7
Ten-pound bowls held alternately 6 hours at 73°F.(23°C.) and 18 hours at 35°F.(2°C.) for 3 days.	0	5.5	5.7	5.5	5.5	4.7	3.9
	6	5.4	5.9	5.5	5.4	4.8	3.3
	24	5.4	6.0	5.5	5.4	4.9	3.4
	30	5.4	6.0	5.5	5.4	4.0	3.4
	48	5.5	5.9	5.5	5.5	4.9	3.3
	54	5.4	5.9	5.5	5.4	4.8	3.4
	72	5.4	6.0	5.5	5.4	4.8	3.3
Thirty-pound cans removed from the room at 44°F.(7°C.) and held 12 hours at 71°F.(22°C.).	0		6.7	6.0		5.2	3.3
	12		6.2	5.4		5.1	3.7
Thirty-pound cans prepared and held at 73°F.(23°C.) 20 hours, followed by refrigeration at 46°F.(8°C.) 12 hours and then held at 85°F.(29°C.) 23 hours.	0		6.2	5.4		5.1	3.7
	8		5.3	4.6		5.1	3.3
	20		8.8	....		8.2	4.4
	32		8.5	5.1		8.7	4.4
	55		9.4	4.7		9.4	4.3
Thirty-pound cans prepared and held at 82°F.(28°C.) 24 hours and then refrigerated at 47°F.(8°C.) 24 hours.	0		5.3	5.2		3.8	3.8
	6		5.6	5.4		4.4	4.4
	12		5.7	5.5		4.9	4.6
	24		8.3	6.1		8.2	5.6
	48		9.0	6.1		8.9	5.8

The pH and bacterial content of inoculated and uninoculated components of chicken salad were determined during different holding periods at room temperature. In 3 different lots of salad ingredients, analyzed for pH and bacterial content, there was little variation in the analyses. The averages are shown in Table 2. Both the standard plate and micrococcal bacteria grew early and rapidly in the diced chicken, showing it to be a good media for the growth of bacteria. They did not grow as rapidly in mixtures of chicken and celery with salt, salad dressing and/or pickle relish, even though the pH was only slightly higher in the chicken than in the mixtures of the chicken salad components. It appears that the lag in growth of micrococci in chicken salad may be due to the presence of acetic acid.

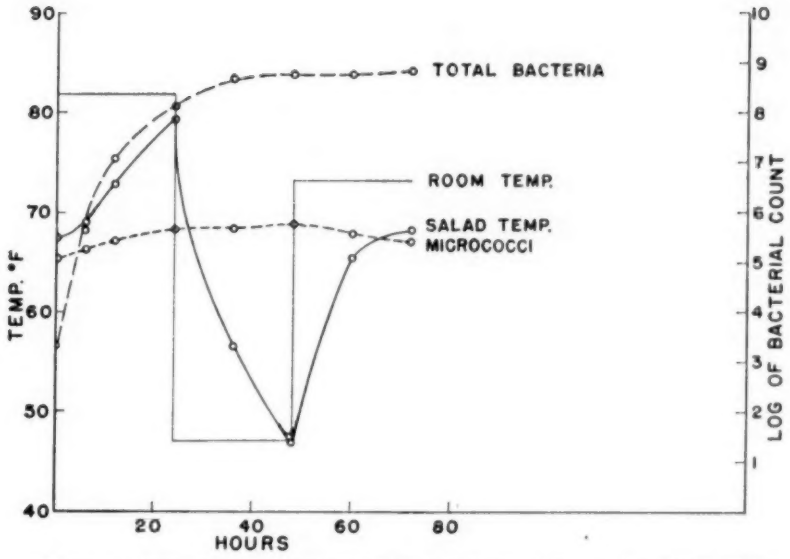

Figure 3. Temperature and bacterial changes in 30 pound cans of inoculated chicken salad held alternately at 82°F.(28°C.) and 47°F.(8°C.) for 72 hours.

Seven strains of *Micrococcus pyogenes* var. *aureus* obtained from food poisoning outbreaks were tested for acid tolerance. Tubes of nutrient broth were prepared with acetic and hydrochloric acid additions to give pH ranges from 4 to 7. The tubes, seeded with 0.1 ml. of a 24 hour broth culture of each of the strains of micrococci, were incubated 48 hours at 37°C.(99°F.). Growth of micrococci was measured by observing the turbidity of the tubes of broth and survival by making stroke plates from the tubes on tryptone glucose extract agar. The strains did not vary in their tolerance.

TABLE 2

Influence of holding conditions on bacterial growth in components of chicken salad

Components of chicken salad	Time held, hrs.	Inoculated			Control		
		Log. of bact. count			Log. of bact. count		
		Standard			Standard		
		pH	plate	Staph.	pH	plate	Staph.
Diced chicken	0		4.9	4.7	3.8-6.2	4.4	4.0
	6		7.0	6.8	5.6-6.2	6.0	5.7
Chicken, salt and celery	0		5.7	4.7	5.6-6.1	5.7	4.0
	6		5.8	5.6	5.7-5.9	5.4	4.7
	24		9.5	9.3	5.6-5.9	8.4	8.2
Chicken, salt, celery, and salad dressing	0		5.3	4.7	5.3-5.7	4.8	3.8
	6		5.4	4.7	5.3-5.6	5.2	3.8
	24		8.0	7.9	4.8-5.8	8.0	7.6
Chicken, salt, celery, salad dressing, and pickle relish (complete salad)	0		5.1	4.5	5.5-5.8	4.3	4.0
	6		5.1	4.4	5.3-5.5	4.2	3.9
	24		6.4	6.2	5.3-5.9	6.5	6.0

Growth was obtained at a lower pH in the presence of hydrochloric acid than in the presence of acetic acid—a result that is in agreement with the work of Wethington and Fabian *(18)* and others who have reported that certain organic acids, especially acetic acid, have special bactericidal properties against the growth of micrococci.

SUMMARY

Micrococcus pyogenes var. *aureus* grows readily in diced chicken held at 70° F. to 80° F.(21° C. to 27° C.). Chicken salad, as prepared in these studies, had a pH of 5.5 to 5.7. This acidity retards the growth of micrococcal bacteria. Acetic acid (vinegar), a commonly used ingredient, is an important factor in retarding the growth of such bacteria. The chicken salad used in these studies could be held at kitchen temperatures as high as 80°F.(27°C.) and for as long as 12 hours without a marked growth of standard plate and micrococcal bacteria.

About 24 hours were required for 30-pound cans of chicken salad to cool below the danger zone (48°F. or 9°C.) when moved from a kitchen at 79°F.(26°C.) to a refrigerator at 47°F.(8°C.). Only about three hours were required for chicken salad in shallow pans to cool below the danger zone under the same conditions.

These studies indicate that cooked and boned chicken should be refrigerated at once in shallow pans at a temperature no higher than 45°F. (7°C.) and as soon as it is used in chicken salad, the salad should be refrigerated in a similar manner, until served.

Acknowledgment

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DEVELOPMENT AND APPLICATION OF OBJECTIVE METHODS FOR QUALITY EVALUATION OF SPICES. I. COLOR-GRADING OF CAPSICUMS[a, b]

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Although the varieties of the genus *Capsicum* differ widely in many characteristics, one criterion of prime quality of members of this genus, especially of the mild or paprika varieties, is redness. As capsicum products are used in foods mainly to impart color, they assume the role of a natural dye. Quality-minded food processors must be able to grade accurately the color of these capsicum products, to insure control of color in their finished goods. In addition, an accurate estimation of color value is of importance to those who buy and sell capsicum spice or oleoresins derived from it.

The method commonly employed for estimation of the color of capsicum spice was suggested in 1910 by Lowenstein and Dunne *(4)*. These investigators prepared alcoholic extracts of paprika and observed the color of the solutions in a Lovibond Tintometer. They noted the number of red units in the presence of a constant number (20) of yellow units. The Lovibond method has all the disadvantages of a subjective technique, and its limitations are well recognized. Furthermore, as will be shown, the relationship between Lovibond red units and pigment concentration is not a linear function, so that all Lovibond measurements must be corrected to obtain true pigment concentration.

While the authors' studies were in progress, Moster and Prater were simultaneously working on the capsicum color problem and have already published their findings *(5)*. Their method is spectrophotometric and is based on the transmittancy readings of an alcoholic extract in a Beckman DU Spectrophotometer at two wavelengths. This method is "more accurate than the Lovibond" technique and, most important, is objective. However, the method is empirical, the relationship between color units and pigment concentration is not linear, transmittancy measurements are made on a steep slope of the transmittancy curve, and expensive equipment is necessary.

In the United States, increasingly greater amounts of capsicum products are being used in the form of oleoresins, usually absorbed onto a salt or dextrose base. The usual method of grading oleoresins *per se* is

[a] Contribution No. 168 from the Department of Food Technology, Massachusetts Institute of Technology. Presented at the Thirteenth Annual Meeting of the IFT, Boston, Mass., June 23, 1953.

[b] Taken in part from a thesis entitled "The Development and Application of Objective Methods for the Quality Evaluation of Capsicum Products," submitted by H. V. Shuster to the Massachusetts Institute of Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, January 1953.

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described by Guenther *(2)*. It is a visual comparison of the "duplication" type, in which a sample is diluted with acetone to match an artificial standard of potassium dichromate and cobaltous chloride when viewed vertically in matched 50-ml. Nessler tubes. The "color value" of the oleoresin is defined as the number of ml. of acetone, multiplied by 100, which must be added to 1 ml. of a 1% solution of the oleoresin in acetone, in order to match the color standard. The standard and the sample are matched for intensity only, as the two solutions do not make a satisfactory color match. Further, in some cases, results are expressed on a 1-gram basis, in others on a 1-ml. basis. These facts, in addition to the inherent subjectivity, offer little recommendation for the method. Here, as is also the case with the ground spice, there is need for an objective, precise, simple, and universal color-grading technique. This paper reports some results from a study that should lead to a method fulfilling these requirements.

BASIS OF METHODS

The color substances of the fruits of the genus *Capsicum* belong chemically to the carotenoids. According to the most recent investigation of this subject *(1)*, the following color substances have been identified in paprika pigment:

capsanthin } red	α carotene } yellow	cryptoxanthin } yellow	
capsorubin }	β carotene }	zeaxanthin	
		xanthophyll	

The bright color of paprika is primarily caused by the red pigments capsanthin and capsorubin. The other color compounds play only a subordinate role. The two red pigments, especially capsanthin, also predominate quantitatively. Their tinctorial power is about 10 times as great as that of the yellow components.

As the spectral properties of the red pigments are so strikingly similar, it is reasonable to assume that the combination would fix the spectral properties of the total paprika pigment. If the total pigment were extracted as a unit, it might be ideally suited to spectrophotometric measurement and be directly related to the true quantitative color value of all types of capsicum spice and oleoresin. All available evidence indicates that the pigments in the two species of *Capsicum, annum* and *frutescens*, are identical although their relative proportions may vary.

EXPERIMENTAL METHODS AND RESULTS

Oleoresins of paprika and red pepper were investigated first, because the color pigments in these materials are directly available and require no extraction prior to examination. Statistical evaluation of the Guenther method *(2)* did not show any difference to exist between the precisions of a trained and an untrained analyst but it did show that there is a greater discrepancy between the results of different observers than between the results of one and the same observer. Thus, when 7 analysts determined the color value of a sample of oleoresin paprika in triplicate according to the Guenther method, their color values ranged from 15,500 to 21,300. When one experienced analyst determined the color values of ten different extracts of the sample, his color values ranged from 15,500 to 17,800.

One-gram (1.0000) samples of 13 different oleoresins were diluted 1:10,000 with acetone (except sample 7, which was diluted 1:2,000), and the visible transmittancy spectra (400-700 mμ) were prepared on the Hardy Recording Spectrophotometer with the use of 1-cm. cells and a slit width of 10 mμ (Figure 1). The curves are fairly similar in shape, the minimum transmittancy exhibiting a mean wavelength of 462 ± 1 mμ. In most cases the transmittancy at the minimum is constant between 452-453 and 470-471 mμ. The Hardy instrument was found equally as satisfactory as the Beckman DU Spectrophotometer for measuring small differences in concentration in these materials.

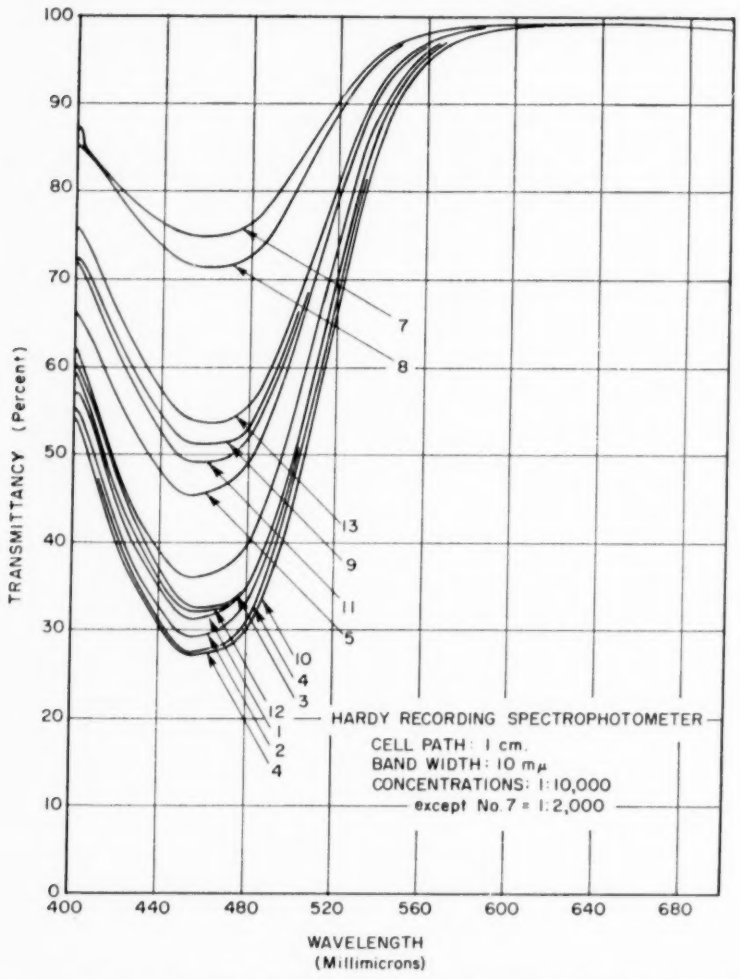

Figure 1. Visible spectra of commercial paprika and capsicum oleoresins, determined on the Hardy Recording Spectrophotometer (cell path, 1 cm.; band width, 10 mμ; concentrations, 1:10,000 except for sample 7 where concentration was 1:2000).

Various filters and filter combinations were used with the Ceneo-Sheard-Sanford Photelometer, and their sensitivities in measuring differences in pigment concentrations in the oleoresins were compared with the sensitivity of the Beckman DU Spectrophotometer. The most sensitive filter combination found was the Evelyn 470 filter,[4] which provided a sensitivity of 95% as compared with that of the Beckman instrument.

[4]Supplied with the Evelyn Photoelectric Colorimeter (Rubicon Company, Philadelphia, Pa.). It is a combination of Corning glasses Nos. 3387, 4303-2, and 5113-1. The spectral properties of this filter combination are maintained remarkably constant by the manufacturer.

Several interference filters of the Fabry-Perot type were tried, but the best of them was no better than the Evelyn 470 filter. Although the interference filters used exhibit an extremely narrow band pass, they transmit slightly over a large range on either side of the peak, especially on the low wavelength side.

The color values of the 13 oleoresins previously examined and the color value of one additional sample were determined in triplicate by the Guenther method and, with the use of the Evelyn 470 filter, in duplicate by the photometric method. The results are compared in Table 1. The correlation between color values as determined by the two

TABLE 1

Determination of relative pigment concentration of oleoresins paprika and capsicum by the Guenther and the photometric methods

Sample No.	Designation	Color value (Guenther)	Absorbancy of undiluted oleoresin, per cm. path[1]
7	Capsicum	1,510	214
8	Capsicum	10,460	1,400
13	Paprika	16,400	2,250
9	Capsicum	18,530	2,470
11	Paprika	19,770	2,760
5	Paprika	20,450	2,910
6	Paprika	26,690	3,900
3	Paprika	31,820	4,300
1	Paprika	31,150	4,520
12	Paprika	31,820	4,560
2	Paprika	33,610	4,630
4	Paprika	33,610	5,000
10	Paprika	33,610	5,050
14	Paprika	46,060	6,570

[1]Cenco-Sheard-Sanford Photelometer; Evelyn 470 filter.

methods is significant at the 0.1% level. The calculated correlation coefficient is 0.996.

On the basis of these results, the investigation was extended to ground capsicum spice. The first step was to extract the maximum amount of carotenoid pigment from the ground spice. Seven solvents* were compared: ethanol (both 190 proof and absolute), anhydrous ethyl ether, acetone, benzene, chloroform, methanol, and carbon tetrachloride. It was planned to use the same capsicum extract to measure both color and pungency. Capsaicin, the pungent principle, is readily soluble in these 7 solvents. The location and fine structure of the absorption maxima are strongly dependent on the solvent. The solvent also has some influence on the magnitude of absorbancies. However, the influence of the solvent was not taken into consideration; only the gross pigment intensity was measured. Samples of commercial, ground, Spanish paprika were weighed out accurately in 100.0-mg. amounts, made to 100 ml. with solvent in volumetric flasks, and macerated 26 hours in a bacteriological incubator at 37°C.(98.6°F.). Macerates were shaken when made up and again one hour before examination. The chloroform and carbon tetrachloride macerates were impractical to handle, for the spice floated on the surface and the macerates had to be filtered prior to examination. With the other solvents, extracts could be pipetted out. Ethyl ether, because of its extreme volatility, was considered unsuitable. Transmittancies of the extracts were determined on the Hardy Recording Spectrophotometer, to locate the median wavelength of the transmittancy minimum. The transmittancies of the highest color macerates were then determined at that wavelength on the Beckman instrument (Table 2).

Other extraction techniques were found to be less efficient than maceration. Within wide limits, size of sample had no effect on efficiency of extraction.

*Mallinckrodt, Analytical Reagent Grade, except for the ethanol which was manufactured by the U. S. Industrial Chemicals Company.

TABLE 2

Relative efficacies of solvents in extracting color pigment from ground Spanish paprika

Solvent	Median wavelength (mμ)	Average absorbancy[1]
Acetone	462	0.618
Methanol	458	0.608
Ethanol (96%)	462	0.583
Ethanol (absolute)	462	0.581

[1]Beckman DU Quartz Spectrophotometer (slit width, 0.030 mm.; cell path, 1.000 cm.; concentration, 1:1,000).

The effects of time, temperature, and particle size on extraction rates were studied. Domestic paprika pods were sorted to remove stems, calyxes, and seeds. The sorted pods were ground in a Wiley Mill to pass a 2.0-mm. screen. Portions of this material were reground to pass 1.0- and 0.5-mm. screens, respectively. Samples of these 3 particle sizes were placed in a sealed desiccator (without desiccant) and left there for 12 days in the dark to eliminate any differences in moisture content. The samples were then macerated with acetone as before for 6, 12, 24, and 48 hours at temperatures of 22° to 23°C.(72° to 73°F., room temperature), 37° ± 2°C.(ca. 98.6°F.), and 46° ±2°C.(ca. 113°F.). Readings were made on the Beckman instrument at the end of the periods specified (Table 3).

TABLE 3

Effects of time, temperature, and particle size on extraction of color pigment from ground paprika by maceration with acetone

Temperature (°C.)	Absorbancy[1] at 464 mμ after maceration for			
	6 hours	12 hours	24 hours	48 hours
0.5-mm. mesh				
22-23	0.486	0.494	0.498	0.500
37	0.488	0.494	0.494	0.493
45	0.484	0.495	0.496	0.492
1.0-mm. mesh				
22-23	0.470	0.483	0.490	0.498
37	0.479	0.488	0.496	0.496
45	0.477	0.484	0.490	0.487
2.0-mm. mesh				
22-23	0.441	0.466	0.484	0.498
37	0.443	0.465	0.483	0.491
45	0.476	0.484	0.492	0.492

[1]Beckman DU Quartz Spectrophotometer (cell path, 1.009 cm.; slit width, 0.035 mm.; concentration, 1:2,000).

The ability of untrained observers to grade paprika samples visually was examined. Samples were lightly packed, as uniformly as possible, in aluminum cups 20 mm. in diameter and 8 mm. deep. They were viewed against a white background, artificial lighting being provided by "cool-white" fluorescent bulbs. These bulbs have an energy distribution closely approximating that of daylight. Observers were asked to arrange samples in order of increasing redness. They were cautioned not to regard the presence of yellow or brown components as redness, for these components tend to mask the redness by alteration of the hue. Observers were also asked to assign arbitrary grades to the samples based on visual examination, assuming the *least red* to have a grade of 1 and the *reddest* a grade of 25.

Each of 10 observers graded a series of 12 samples of Spanish paprika twice, at an interval of 1 week. The correlation between the 2 rankings was evaluated statistically by use of the "rank correlation." This correlation for all observers was sig-

nificant at the 0.1% level. The color pigment was extracted from the samples by maceration with acetone, and the visible spectra were read on the Hardy instrument. The transmittancy curves agreed well with those of Figure 1. A large number of experiments confirmed the fact that the character of visible transmittancy curves of acetone extracts of paprika is independent of the source of the spice. Absorbancies at 462 mμ correlated significantly (0.1% level) with visual scores (Table 4).

A series of 12 samples of domestic paprika was graded both visually and spectrophotometrically, as above. These samples were from the same grower and were identical in particle size, age, history, and moisture content. Agreement between observers was better than was the case with the Spanish paprika samples, due undoubtedly to control of the above variables. The grower had previously graded the samples by the usual technique of measuring ethanolic extracts on the Lovibond Tintometer. He informed the authors that the red coloring power of a highly red material is greater, when compared with a material of lesser red value, than a simple ratio of Lovibond red values

TABLE 4

Comparison of visual score and absorbancy of acetone-extracted pigment of ground Spanish paprika

Sample No.	Visual score[1]	Absorbancy[2] at 462 mμ
A	25.0	0.828
I	22.0	0.809
B	17.4	0.768
G	13.4	0.710
C	17.3	0.703
L	18.5	0.616
D	10.1	0.533
K	9.9	0.531
J	3.8	0.506
F	6.2	0.501
H	3.3	0.480
E	1.0	0.455

[1] Average of 10 observers.
[2] Absorbancy determined with Hardy Recording Spectrophotometer (band width, 10 mμ; cell path, 1 cm.; concentration, 1:1,000).

TABLE 5

Composite grading data on domestic paprika samples

Sample No.	Visual score[1]	Lovibond red[2]	C-units[3]	Absorbancy at 462 mμ[4]	Order[5]
1	24.7	17.7	44.6	1.024	1
2	23.9	16.8	41.0	0.950	2
3	21.7	15.6	36.5	0.870	3
4	19.3	15.0	34.0	0.830	4
5	16.1	14.6	32.6	0.752	5
6	12.2	13.7	29.5	0.676	6
7	11.8	13.3	27.7	0.620	7
8	8.2	11.9	23.8	0.540	8
9	6.0	10.8	20.7	0.485	9
10	3.9	9.6	17.4	0.415	10
11	2.2	8.2	14.0	0.342	11
12	1.0	7.0	11.0	0.277	12

[1] Average of 10 observers.
[2] 2 in. cell. Lovibond yellow units constant at 20-27; concentration, 1:500; ethanolic extract.
[3] 2-in. cell. From Lovibond red units.
[4] Hardy Recording Spectrophotometer (cell path, 1 cm.; band width, 10 mμ; concentration, 1:1,000; acetone extract).
[5] An order of 1 signifies the reddest sample. The order was the same for the visual scores, the absorbancies, and the C-units.

would indicate. For example, a 24.0 Lovibond red material diluted two-fold will give a reading higher than 12.0 Lovibond red. This observation has been confirmed. Using 20.0 Lovibond red as a base point and increasing or decreasing concentrations from this point, the grower was able to make a straight-line plot between Lovibond and "corrected" units. Relative pigment concentration is then measured in terms of "C" units.

Composite data for the domestic paprika samples are presented in Table 5. A linear plot results if C units are plotted against absorbancies of acetone extracts at 462 mμ (Figure 2).

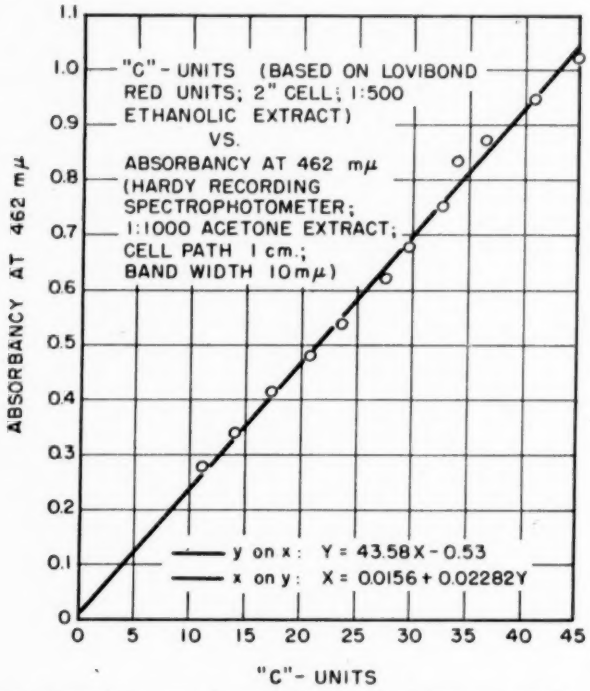

Figure 2. Regression curves of extract absorbancy of domestic paprikas against "C" units (based on Lovibond red units; 2-in. cell; 1:500 ethanolic extract) vs. absorbancy at 462 mμ (Hardy Recording Spectrophotometer; 1:1000 acetone extract; cell path, 1 cm.; band width, 10 mμ).

A group of 20 paprikas of several origins and degrees of freshness were examined by the Lovibond method and by the photometric method. Both the Beckman instrument and the Ceneo-Sheard-Sanford photelometer with the Evelyn 470 filter were used. When the Beckman readings were used as the standard for comparison, the photelometer method was found to measure small differences in pigment concentration more accurately than the Lovibond technique (Table 6).

DISCUSSION

The transmittancy curves obtained for the oleoresins studied (Figure 1) are characteristic of curves obtained for the color pigment of capsicum spices. The curve for sample 7, typical of a fresh sample, exhibits a flat

TABLE 6

Composite grading data for paprika samples

Sample No.	Photelometer[1]	Absorbancy, Beckman[2]	C units[3]
5	0.103	0.120	5.7
2	0.133	0.164	12.9
19	0.249	0.270	22.7
15	0.247	0.271	21.9
9	0.238	0.271	22.1
12	0.245	0.274	22.8
16	0.255	0.275	24.1
1	0.260	0.295	24.8
10	0.268	0.300	23.5
11	0.269	0.302	24.1
3	0.268	0.303	24.0
14	0.280	0.313	24.8
8	0.280	0.317	24.0
18	0.280	0.317	24.0
20	0.305	0.338	30.5
4	0.298	0.338	25.7
13	0.309	0.339	32.5
17	0.322	0.362	29.6
7	0.402	0.453	33.4
6	0.478	0.533	42.3

[1] 1:2,000 acetone extract; Cenco-Sheard-Sanford Photelometer; Evelyn 470 filter; cell path, 1.0 cm.
[2] 1:2,000 acetone extract; slit width, 0.025 mm.; cell path, 1.000 cm.
[3] Converted from Lovibond red tintometer readings; 5¼ in. tube; average of 2 observers.

minimum which, when determined by the Beckman Spectrophotometer, usually extends over a band of approximately 18 mμ. As a sample ages or is exposed to heat or light, the natural trans-carotenoids isomerize, the minimum shifts to shorter wavelengths (e.g., sample 5) and the curve at these wavelengths loses its flat shape. However, the median wavelength at which minimum transmittancy occurs conveniently remains remarkably constant at 462 mμ. A spectrophotometric or photometric reading then measures the average level of the slope at the transmittancy minimum.

According to the data in Table 1, the photometric color values of the oleoresins examined reflect perfectly the Guenther color values, within the limits of precision of the latter method. One of the companies from which the oleoresins were obtained expressed Guenther values on a 1-ml. basis. These values bore no relation to the concentration of pigment in the material, even when the values were corrected to a 1-g. basis.

A study of the efficacies of various solvents in extracting color pigment from Spanish paprika shows that the character of the transmittancy curve (on the Hardy instrument) is independent of the solvent. The location of the minimum, however, is dependent on the solvent. When read on the Beckman instrument, the *fine* structure is dependent on the solvent.

Acetone extracted essentially all the carotenoid pigment. Redistilling the acetone from the extract did not affect its extraction properties. The plateau-like absorbancy maximum of the acetone extract was fortuitous and lent itself perfectly to spectrophotometric measurement. Frequently the visible spectra of ethanolic extracts were not clearly resolved. This was probably due to the interaction which can occur between the sol-

vent and the carotenoids containing carbonyl groups (e.g., capsanthin, capsorubin).

As would be expected, the rate of color extraction is dependent upon temperature and particle size (cf. Table 3). The particle sizes of the majority of commercial samples are equivalent to or smaller than 0.5-mm. mesh. The highest absorbancies were obtained in extracts ground to pass through a sieve of 0.5-mm. mesh. The data in Table 3 also show that maceration for periods greater than 24 hours at 45°C.(113°F.) caused a slight reduction in the concentration of color pigment. On the basis of these results, a maceration time of 24 hours, a temperature of 37°C.(98.6°F.), and a mesh size of 0.5 mm. or smaller were chosen as the best conditions for maximum extraction of color. The precision of color extraction with acetone under these conditions was high. For example, when ten portions of the same sample were extracted, and the colors were measured in the photelometer with the Evelyn 470 filter, the 95% confidence limit (single determination) of absorbancy was 0.397 ± 0.004. The extractable pigment concentrations conformed to Beer's Law within the workable range.

The good correlation between visual scores and tinctorial values (Table 4) confirms the empirical observation of many food processors. Samples that were old and had brownish hues were extremely difficult to grade visually, and the scores for these samples varied greatly between observers. However, the acetone extracts of these samples displayed hues identical with those of the fresh materials. Water extracts (1:100) of the brownish samples, when filtered clear, showed visible absorbancy curves similar to those of aqueous extracts of non-enzymatically browned dehydrated vegetables *(3)*.

The regression curve of Figure 2 shows a correlation between absorbancy and C-units that is significant at the 0.1% level. Lovibond red values could be read from this plot by use of the spectrophotometric values. However, the Lovibond red values would apply only to the observers in question, for the sensitivities of observers vary within wide limits.

According to Table 6, the photelometer method is relatively more sensitive than the Lovibond technique and permits more accurate measurement of small differences in color of paprika extracts than does the latter. It is important to remember that the Lovibond technique measures concentration at a wavelength far removed from the maximum absorbancy where small differences in concentration are detected most precisely. In these studies it was found that the effect of the chlorophyll on the measurement of color concentration was negligible and within the experimental error. In this respect the spectrophotometric method has a distinct advantage over the Lovibond technique in which the green pigments may play a significant role.

From the results obtained it would seem that the Beckman DU Quartz Spectrophotometer would be the instrument of choice in grading samples of the genus *Capsicum*, especially paprika varieties. This spectrophotometer is easily calibrated[1] and gives the most sensitive measurements of concentration and differences in concentration with a single reading at 462

[1] National Bureau of Standards, Letter Circular LC 929, Nov. 26, 1948.

mμ. However, for routine quality control, it is not always available. For routine work, a simple photometer of the Cenco-Sheard-Sanford type can be used with the Evelyn 470 filter combination. An even less refined photometer would suffice.

One problem has yet to be solved completely. Absorbancy readings on a photometer will vary according to the type of instrument used and also among different instruments of the same type. Some of the variable factors are the optical system, the electrical system, the cuvette thickness, and photocell variables. Use of a standardized Evelyn 470 filter would eliminate differences in band widths and in the characteristics of filter transmission. To define pigment concentration in terms of absorbancy, all the above variables would have to be specified, which in turn would require practically the designation of a master instrument against which all other instruments would have to be calibrated by some means.

The best solution of the problem entailed in the use of a photometric method is to define color in terms of the concentration of a colored material. This material should ideally have a visible transmittancy curve identical with that of the capsicum acetone extract, should be stable and non-hygroscopic, and should follow Beer's Law over the required range. Organic dyestuffs offer the best possibility. The writers have found several materials that approximate the above requirements. A report on color properties of selected dyestuffs and their use as standard materials for photometer calibration will be forthcoming shortly.

SUMMARY

The precision of existing visual methods for the color grading of oleoresins of the genus *Capsicum* has been evaluated. The visible spectra of acetone solutions of these oleoresins were essentially identical, exhibiting a flat transmittancy minimum ca. 18 mμ in width with a median wavelength of 462 ± 1-2 mμ. Differences in color values of oleoresins were precisely determined with the Beckman Model DU Spectrophotometer by a single reading at 462 mμ or with the Cenco-Sheard-Sanford photelometer with the Evelyn 470 filter combination. Photometric values correlated linearly and significanfly (0.1% level) with visual comparison values.

The color grading of ground spice of the genus *Capsicum* has been investigated, and special emphasis has been given to paprika varieties. A technique has been determined for extraction of maximum carotenoid pigment from the spice by maceration with acetone. The error of the technique is approximately one per cent.

Extraction rates were dependent upon time of maceration, temperature, and particle size. Visible transmittancy curves agreed with those of oleoresins of the genus. Extracts of samples that had been in extended storage absorbed at shorter wavelengths in the visible region. The acetone extracts followed Beer's Law within the workable range.

Spectrophotometric absorbancies of acetone extracts at 462 mμ correlated significantly (0.1% level) with visual scores (10 observers) and also with Lovibond red values (corrected to a straight-line dilution curve) of ethanolic extracts. The absorbancies were determined with high sensitivity, by use of filter photometry.

Color evaluation of acetone extracts by either spectrophotometer or filter photometry was superior to a Lovibond red description in distinguishing small color differences.

The practical application of these studies as related to objective color grading of capsicum spices and oleoresins is discussed.

Acknowledgment

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THE EFFECT OF SEVERAL ANTIBIOTICS ON CERTAIN SPORE-FORMING ORGANISMS INVOLVED IN FOOD SPOILAGE[a]

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Food preservation in canning is accomplished by the thermal destruction of the deleterious organisms which may be present. Acid foods having a pH below 4.5 are conventionally processed in boiling water under atmospheric pressure; the heat resistant spores associated with these products are, in general, readily destroyed during the heat treatment or will not grow under such acid conditions.

In some acid foods, however, this conventional process has failed to control spoilage in several instances. Townsend *(16)* isolated acid-tolerant, mesophilic, sporeforming anaerobes from several outbreaks of spoilage in various canned fruit and tomato products. A similar outbreak has been reported in canned pineapple juice *(16)*. The organisms isolated had a thermal death time of 90 minutes at 203°F.(95°C.) in tomato juice at pH 4.5 and grew in natural fruit juices below pH 4.5. From spoiled canned tomato juice, Berry *(1)* isolated an aciduric, thermophilic, sporeforming, facultative anaerobe which he named *B. thermoacidurans*. The spores of this organism had a z value of 14.5°F.(8°C.) and an F value of 0.33 minutes in tomato juice at pH 4.5. They also germinated and grew in tomato juice at pH 4.3 *(14)*. A high temperature short time process *(6)* at 121°C. (250°F.) for 0.7 of a minute has been recommended to prevent this type of spoilage; White *(17)*, however, reported typical *B. thermoacidurans* spoilage in two plants using this process. Furthermore, Pederson and Becker *(14)* pointed out that some canneries are not equipped for processing at this high temperature.

Medium and nonacid foods *(11)* having a pH above 4.5 are conventionally processed under high pressures and temperatures. This technique, however, has several limitations. Spores of some of the spoilage organisms, generally associated with these food products, are among the most heat resistant organisms known and make absolute sterility practically impossible without impairing the quality of the product or rendering it unfit for human use *(15)*. Meticulous sanitary procedures, adequate cooling, and proper storage temperature are the only positive measures taken to prevent the growth of the surviving organisms; at times, these measures are not controlled properly or are impossible to maintain under peak operating conditions and serious spoilage outbreaks result.

Because of the above limitations in the thermal processing of both acid and low acid foods, many antibacterial agents have been investigated in an attempt to find some which might destroy or reduce the thermal resistance of the spores and thereby eliminate the necessity for severe heat treatment. Use of antibiotics to achieve this objective has been investi-

[a]This work was supported by the University of Illinois Research Foundation.

gated by several workers. Curran and Evans *(8, 9)* and Burroughs and Wheaton *(7)* showed penicillin to have no effect against *Cl. botulinum* or *Cl. sporogenes* (PA 3679) in milk or corn; the latter concluded that penicillin was of no value in food preservation. Andersen and Michener *(2, 3)* found that subtilin and mild heating controlled the spoilage of corn, peas, and asparagus in both uninoculated and inoculated packs using *B. stearothermophilus* (FS 1518), *Cl. botulinum* and *Cl. sporogenes* (PA 3679) as test organisms. Burroughs and Wheaton *(7)*, and Bohrer *(5)* demonstrated that subtilin, gramicidin, methylol gramicidin, bacitracin, streptomycin, and terramycin did not destroy the spores of *Cl. botulinum* or *Cl. sporogenes* (PA 3679) in peas, corn, chicken soup, onion, cauliflower, broccoli, celery, or sweet potatoes. In a few instances subtilin did exert some sporostatic effect against the spores of these organisms. It was also shown *(5, 7)* that subtilin apparently destroyed the spores of *B. stearothermophilus* (FS 1518) and *B. thermoacidurans*. Olcott *(12)* found that subtilin was ineffective againt *Cl. sporogenes* (PA 3679) in pea puree. Subtilin, streptomycin, penicillin, and terramycin have also been shown by Ordal *(13)* to be of no value in controlling the growth of *B. stearothermophilus* (FS 1518) in corn and peas. Adams, Ayers, and Tischer *(1)* have reported that *Cl. sporogenes* is not inhibited in comminuted beef containing subtilin. Williams and Campbell *(18)*, using 30 strains of sporeforming obligate and facultative thermophilic flat sour bacteria, concluded that subtilin and mild heating was ineffective for complete destruction of the spores. Recently, however, LeBlanc, Devlin, and Stumbo *(10)* reported that the heat resistance of spores of *Cl. sporogenes* (PA 3679) and *Cl. botulinum* is appreciably lowered in pea puree containing small amounts of subtilin.

This investigation was, therefore, set up to screen 9 antibiotics against 5 sporeforming organisms commonly involved in the spoilage of canned foods. Each antibiotic was tested against each organism.

EXPERIMENTAL

The antibiotics neomycin,[b] celiomycin,[c] streptin,[c] circulin (Q-19),[d] M-2517-6 (911-A),[e] 51R4076 (A-20),[c] M-3461-A (HE-833-A),[e] M-3385 (HE-922-A),[e] and M-3478-3 (HE-900-A)[e] were tested against *B. thermoacidurans* (43-P), *B. stearothermophilus* (FS 1518), *Cl. botulinum* (Type B), *Cl. sporogenes* (PA 3679), and *Cl. thermosaccharolyticum* (TA 3814). For assay purposes the medium was tubed in 9-ml. portions. Spores of *B. thermoacidurans* were grown on thermoacidurans agar (Difco) for 5 to 7 days at 131°F.(55°C.). It was observed in our laboratory that keeping the surface of the agar as dry as possible enhanced sporulation considerably. Spores of *B. stearothermophilus* were readily obtained on nutrient agar in 3 days at 131°F.(55°C.). All spore harvests were washed three times and suspended in sterile distilled water for use as stock spore suspensions. The stock spore suspensions were heated in flowing steam for 8 minutes to destroy vegetative cells. With the anaerobes, 0.2 ml. of a 48-hour broth culture was

[b] Neomycin sulfate (Rx 9291) was obtained through the courtesy of Dr. D. R. Colingsworth, The Upjohn Co.

[c] Celiomycin hydrochloride-51R3162, crude-51R4076 (A-20), and Streptin crude-51R4393, were obtained through the courtesy of Dr. H. B. Woodruff, Merck and Co., Inc.

[d] Circulin Q-19, Res. No. 8836 was obtained through the courtesy of Dr. H. Koffler, Purdue University.

[e] These antibiotics were obtained through the courtesy of Dr. J. F. Pagano, E. R. Squibb & Sons.

used as an inoculum. In preparing the assay medium for *Cl. thermosaccharolyticum* the liver particles were omitted from the recommended medium in order to obtain a clear solution in which growth could easily be detected. Stock solutions containing 10,000 γ per ml. of each antibiotic were prepared and sterilized by Seitz filtration. These stock solutions were diluted serially in sterile distilled water to a level of 0.1 γ per ml. and stored under refrigeration.

In the assay procedure, one ml. of the antibiotic solution was added to the test medium prior to inoculation. Growth and sterility controls were included and all assays were done in triplicate. All cultures were incubated at their optimum temperature for 7 days, stored for 20 days at room temperature, reincubated for 5 days, stored again for 20 days at room temperature, and reincubated again for 4 days. Turbidity in the test medium was used to indicate growth. Positive tubes were examined microscopically for typical growth of the test organisms. Negative tubes were subcultured in antibiotic-free medium at the end of the 8-week test period to detect viable cells. In tubes containing high levels of antibiotic, it was necessary to dilute the transfer to a noninhibitory level; subcultures were examined for growth after a 2-week incubation period.

RESULTS AND DISCUSSION

The final inhibitory endpoints obtained in each case are summarized in Table 1. The data obtained on subculturing the negative tubes from the screening tests, showing those instances in which viable organisms were recovered are presented in Table 2. It is interesting to note that all the antibiotics except crude M-51R4076 (A-20) inhibited the spores of *B. stearothermophilus* (FS 1518) at low concentrations. Data in Table 2 show that no viable organisms could be recovered from negative tubes and suggest that these antibiotics are sporocidal for this organism. The sensitivity of *B. stearothermophilus* strains to antibiotics is not new; other investigators *(2, 3, 6, 9, 10, 13, 14, 20)* have shown that this organism is also inhibited by penicillin, subtilin, terramycin, streptomycin, and chloromycetin at relatively low concentrations. In most of these cases, however, the effect was merely sporostatic.

TABLE 1

Inhibitory concentrations of several antibiotics against certain food spoilage organisms at the end of eight weeks

Antibiotics	Inhibitory concentrations in γ per ml.				
	B. thermoacidurans 43-P	*B. stearothermophilus* FS 1518	*Cl. botulinum* Type B	*Cl. sporogenes* PA 3679	*Cl. thermosaccharolyticum* TA 3814
Neomycin	1.0	0.2	250	>1000	100
Celiomycin	62.5	7.8	>1000	>1000	31.3
Streptin	500	7.8	>1000	>1000	>1000
Circulin (Q-19)	7.8	1.0	>1000	>1000	>1000
M-2517-6 (911-A)	15.6	0.5	>1000	>1000	>1000
M-51R4076 (A-20)	>1000	>1000	>1000	>1000	>1000
M-3461-A (HE-833-A)	62.5	0.5	>1000	>1000	>1000
M-3385 (HE-922-A)	250	1.0	>1000	>1000	>1000
M-3478-3 (HE-900-A)	>1000	1.9	>1000	>1000	>1000

Neomycin, celiomycin, and M-2517-6 (911-A) inhibited *B. thermoacidurans* (43-P) at 1, 62.5, and 15.6 γ per ml., respectively, after an 8-week test period. At these concentrations no growth was observed in the

subcultures made from negative tubes (Table 2) which also suggests a sporocidal action. Circulin, M-3461-A (He-833-A), M-3385 (HE-922-A) and streptin inhibited this organism in concentrations ranging from 7.8 to 500 γ per ml. but with these antibiotics the inhibited spores grew on subculturing (Table 2).

Neomycin and celiomycin exerted some bactericidal action against *Cl. thermosaccharolyticum* (TA 3814) at a concentration of 100 and 31.3 γ per ml., respectively. Neomycin appeared to be bacteriostatic for *Cl. botulinum* (Type B) at a concentration of 250 γ per ml.; none of the antibiotics, however, showed any activity against *Cl. sporogenes* (PA 3679).

TABLE 2

Recovery of the test organisms as indicated by turbidity on subculturing

Antibiotics	Recovery of organisms shown			
	B. thermoacidurans 43-P	*B. stearothermophilus* FS 1518	*Cl. botulinum* Type B	*Cl. thermosaccharolyticum* TA 3814
Neomycin	—[a]	—	+[b]	—
Celiomycin	—	—	...[c]	—
Streptin	+	—	...	...
Circulin (Q-19)	+	—	...	...
M-2517-6 (911-A)	—	—	...	...
M-3461-A (HE-833-A)	+	—	...	...
M-3385 (HE-922-A)	+	—	...	...
M-3478-3 (HE-900-A)	...	—	...	...

[a] — represents no recovery on subculturing of all negative tubes.
[b] + represents recovery on subculturing of all negative tubes.
[c] ... Represents ineffective antibiotic.

SUMMARY

Nine antibiotics were screened against 5 sporeforming organisms commonly associated with the spoilage of canned foods. Under the conditions of the test all of the antibiotics were sporocidal for *B. stearothermophilus* (FS 1518) except the crude M-51R4076 (A-20). Neomycin, celiomycin, and M-2517-6 (911-A) had a sporocidal effect against *B. thermoacidurans* (43-P). Neomycin and celiomycin exerted some sporocidal action against *Cl. thermosaccharolyticum* (TA 3814). None of the antibiotics was effective against *Cl. botulinum* (Type B) or *Cl. sporogenes* (PA 3679).

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THE ACTION OF SEVERAL ANTIBIOTICS ON THE SPORES OF *BACILLUS THERMOACIDURANS* IN A TOMATO JUICE MEDIUM[a]

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In the course of an antibiotic screening study *(2)* it was observed that, in proteose peptone broth, neomycin, celiomycin, and M-2517-6 (911-A) inhibited the growth of *B. thermoacidurans* (43-P), an organism responsible for flat sour spoilage in tomato juice. Although this type of spoilage is sporadic, it is of considerable importance as it is impossible to detect the defect without opening each can. Since this is impractical, a spoiled product could reach the consumer and create an unfavorable reaction both to the product and to the packer.

Pederson and Becker *(6)* have shown that the growth of *B. thermoacidurans* is markedly affected by the pH of the tomato juice. They showed that the vegetable cells of this organism grew in juice at pH 4.1; the spores in 20 out of 21 strains, however, did not germinate in juice below pH 4.32. In view of this, attempts have been made by both natural and artificial methods to control the spoilage by maintaining the pH below 4.1. Controlling the pH of the fruit by natural methods has proved impractical as Lee and Sayre *(4)* have shown that the plant variety, the soil moisture content, the type of fertilizer, and the maturity of the plant affect the acid content of tomato juice. Artificially controlling the pH by adding citric acid has been suggested but is ineffective from a practical viewpoint. Murdock *(5)* demonstrated the effectiveness of 0.13% added citric acid in inhibiting the growth of *B. thermoacidurans* in broth cultures at pH 4.6, but the presence of 0.34% citric acid naturally present in tomato juice below pH 4.4, failed to inhibit the organism. A high temperature short time heat treatment at 250°F.(121°C.) for 0.75 of a minute has been recommended to control this spoilage; White *(9)*, however, reported several typical spoilage cases in two plants using this procedure.

Since the antibiotics neomycin, celiomycin, and M-2517-6 (911-A) showed some activity against the spores of *B. thermoacidurans* (43-P) in proteose peptone broth cultures, it was decided to investigate the activity of these antibiotics in tomato juice as a culture medium.

EXPERIMENTAL

In view of the findings of Pederson and Becker *(6)* it became necessary first to determine the tolerance of spores of *B. thermoacidurans* strain 43-P to the acidic conditions found in the tomato juice. Natural tomato juice at pH 4.2 and natural tomato juice adjusted to pH 5.0 with sodium hydroxide were inoculated so as to contain 12,000 spores per ml. and incubated at 131°F.(55°C.) and 98°F.(37°C.). After a 7-day incubation period, the tubes were stored at room temperature for an additional 14 days. The development of acidity was used as a criterion of growth.

[a] This study was supported by the University of Illinois Research Foundation.

A two-fold serial dilution assay procedure *(2)* was carried out, using a tomato juice medium and proteose peptone broth for purposes of comparison. The inhibitory level of each antibiotic was determined by observing the pH of the test medium; no change in pH was indicative of no growth or inhibition. The development of *B. thermoacidurans* in the presence of neomycin was also followed quantitatively by plating on thermoacidurans agar. To determine the residual antibiotic activity after exposure to the test organism, the supernatant serum obtained after centrifuging the tomato juice was serially diluted in nutrient broth and reassayed using *B. stearothermophilus* (FS 1518) as this organism showed a high degree of sensitivity to neomycin. A serial dilution of the antibiotic, *per se*, was assayed simultaneously to determine the standard inhibitory level. Incubation was at 131°F.(55°C.) for 48 hours and growth was indicated by turbidity.

The combined effect of heat and neomycin on the spores of *B. thermoacidurans* (43 P) was also investigated. In this work the assay tubes containing tomato juice at pH 5.0 and antibiotic were exposed to flowing steam for 5 minutes after inoculating at a level of 34,000 spores per ml.

RESULTS AND DISCUSSION

Results obtained on culturing *B. thermoacidurans* (43-P) in natural tomato juice are shown in Figure 1. The spores did not germinate in tomato juice at pH 4.2. Raising the pH to 5.0, however, permitted germination and rapid growth; similar findings have been reported by Pederson and Becker *(6)* for other strains of this organism. In view of this the

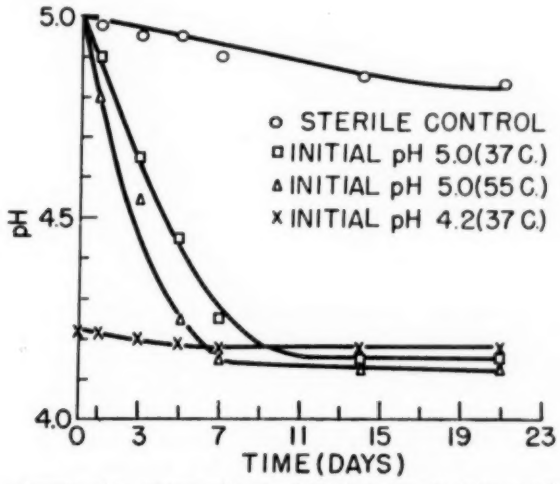

Figure 1. Growth of B. thermoacidurans (43-P) in tomato juice at pH 4.2 and 5.0 as indicated by change in pH.

tomato juice medium was adjusted to pH 5.0 in all studies to obtain luxuriant growth for test purposes.

The inhibitory level of the antibiotics is summarized and compared in Table 1. The activity of celiomycin and M-2517-6 (911-A), using tomato juice at pH 5.0 in place of proteose peptone broth at pH 7.0, decreased to the extent that further studies were discontinued. The activity of neomycin also decreased considerably; at the end of the 28-day test period it

TABLE 1

Inhibitory levels of neomycin, celiomycin and M-2517-6 (911-A) against B. thermoacidurans (43-P) in two media

Antibiotics	Inhibitory levels after 28 days (γ per ml.)	
	Tomato juice (pH 5)	Proteose peptone broth (pH 7)
Neomycin	250	1.0
Celiomycin	>1,000	62.5
M-2517-6 (911-A)	1,000	15.6

was observed that an initial level of 250 γ per ml. was necessary to inhibit growth in tomato juice as compared with 1 γ per ml. in proteose peptone broth.

This decrease in neomycin activity can be explained by an examination of the reassay studies (Table 2) which indicate that the antibiotic activity

TABLE 2

The residual activity of neomycin in tomato juice (pH 5) inoculated with B. thermoacidurans (43-P)

Original concentration of neomycin in tomato juice	Inhibitory dilution of serum after (days)[1]		Calculated residual activity after (days)[2]			
			γ per ml.		%	
	7	42	7	42	7	42
1,000	2^{-14}	2^{-11}	500	62.5	50	6
500	2^{-13}	2^{-10}	250	31.2	50	6
250	2^{-12}	2^{-9}	125	15.6	50	2
62.5	2^{-9}	None	15.6	None	25	0
15.6	2^{-8}	None	1.9	None	13	0

[1] *B. stearothermophilus* was the assay organism.
[2] Calculations were based on a standard inhibitory level of 0.03 γ per ml.

in tomato juice containing initially 1,000, 500, 250, and 62.5 γ per ml. remained constant for 7 days. At the end of 42 days, however, over 90% of the activity was lost. This loss might be attributed to the unfavorable reaction of the tomato juice as Poth *et al. (7)* and Waksman, Lechevalier, and Harris *(8)* have shown that neomycin activity is favored by an alkaline reaction. From the standpoint of a practical canning operation the complete destruction of residual antibiotic on storage would be desirable provided the initial lethal effect was sufficient to destroy all of the organisms present.

The development of *B. thermoacidurans* (43-P) in tomato juice in the presence of neomycin is shown in Figure 2. From this it can be seen that a concentration of antibiotic >250 γ per ml. is bacteriocidal in that, after a 42-day exposure period, no growth was obtained on thermoacidurans agar; the pH also remained unchanged throughout this period. Lower levels of antibiotic (125 to 31 γ per ml.) were only slightly inhibitory as evidenced by the slightly prolonged lag phases. A marked decrease in the initial number of spores was also noted. The spores, however, recovered in these instances and completed a growth cycle similar to the control culture; the maximum level of population was always somewhat less than in the con-

trol. Only slight, if any, inhibitory action was observed at antibiotic concentrations <15.6 γ per ml.

In view of the fact that neomycin, *per se*, showed some activity against the spores of *B. thermoacidurans* (43-P) it was thought desirable to investigate the effect of the antibiotic in combination with a mild heat treatment as Anderson *et al. (1)* and LeBlanc *et al. (3)* have shown that the thermal resistance of several sporeforming organisms is decreased in

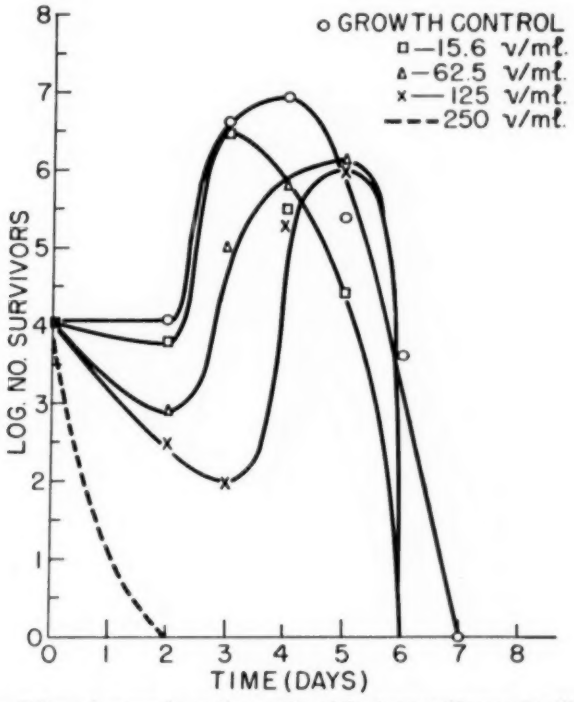

Figure 2. Effect of neomycin on the growth of B. thermoacidurans (43-P) in tomato juice at pH 5.0.

the presence of certain antibiotics. This mild heat treatment would, in addition, serve to destroy enzymatic activity as well as yeasts, molds, and vegetative cells of bacteria which might be resistant to neomycin. Figure 3 shows the growth pattern of *B. thermoacidurans* (43-P) in tomato juice after a 5-minute steaming period in the presence of neomycin. Complete inhibition was not observed even in the presence of 500 γ per ml. of the antibiotic; this level, however, did delay the initiation of growth for 2 days. Inactivation of neomycin is not responsible for the decreased activity as control studies indicate that this antibiotic is not destroyed by heating in tomato juice at pH 5.0. Some explanation for this apparent decrease in activity might be found by comparing the growth control curves with

and without heat treatment in the absence of the antibiotic (Figures 2 and 3). Although exposing the organism to steam for 5 minutes reduced the viable spore count from 34,000 to 750 per ml., the lag phase of growth was also reduced considerably and active growth commenced almost immediately. This is indicated by the fact that after 48 hours the heated sample showed a maximum population level as compared with the unheated

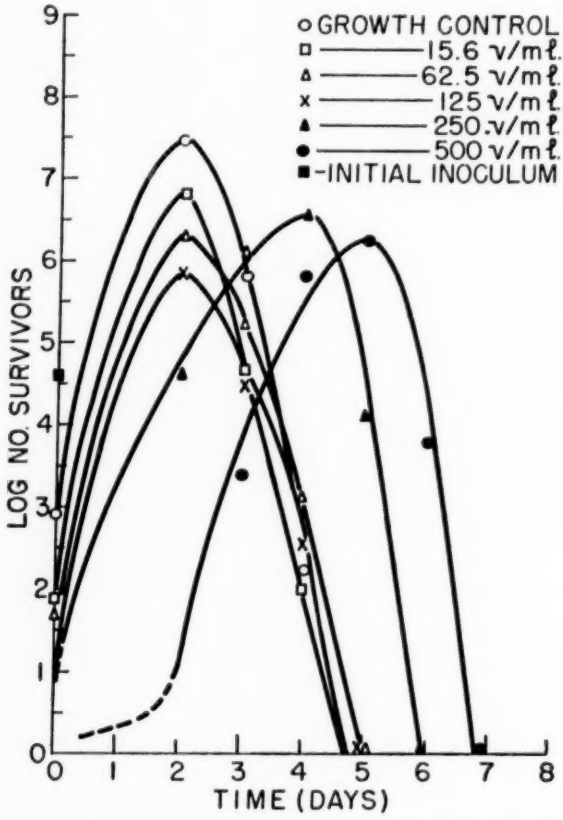

Figure 3. Effect of neomycin and mild heating on the growth of B. thermoacidurans (43-P) in tomato juice at pH 5.0.

sample which showed no increase in number at the end of the same incubation period. It is also interesting that although the initial population was lower in the former, a higher level of growth was obtained. From this it appears that rapid cell proliferation reduced the efficiency of the antibiotic. Since a mild heat treatment is an indispensable technique in any canning operation, the practical application of this antibiotic in canning does not appear feasible.

CONCLUSIONS

An attempt was made to control the flat sour spoilage in tomato juice caused by *B. thermoacidurans* (43-P) using several antibiotics found to be effective against the spores of this organism in proteose peptone broth.

The spores of *B. thermoacidurans* (43-P) did not grow in tomato juice at pH 4.2; tomato juice adjusted to pH 5.0 with NaOH supported the growth of the organism. Both celiomycin and M-2517-6 (911-A) lost much of their activity against the spores when tomato juice at pH 5.0 was used as the test medium. In the presence of the former, growth took place at an antibiotic concentration of 1,000 γ per ml.; in the latter this same concentration exhibited an inhibitory effect. Neomycin, at a concentration of 250 γ per ml. prevented growth of the spores in unheated juice, but was ineffective when the juice was heated in flowing steam for 5 minutes after inoculation and addition of the antibiotic.

Since a mild heat treatment is necessary in order to destroy molds, yeasts, and vegetative cells of bacteria as well as to inactivate enzymes, the present technique seems to have no practical value in the control of this type of spoilage.

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SOURCES AND TYPES OF AEROBIC MICROORGANISMS ASSOCIATED WITH THE SOFTENING OF FERMENTING CUCUMBERS[a,b]

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Bacteria of the genus *Bacillus* in the past generally have been regarded as the chief cause of the softening of fermenting cucumbers. However, it is quite possible that this spoilage results from the activity of pectolytic enzymes elaborated by yeasts *(13)*, molds *(16)*, cucumbers *(3)*, or, perhaps, a combination of enzymes from these several sources. The exact cause still is controversial.

It was thought that a survey to determine the sources and types of microorganisms which might be associated with softening would provide additional information of value for elucidation of the etiology of this spoilage. An opportunity to make such a survey arose in connection with the study of a commercial outbreak of softening. The results pertaining to the sources and types of aerobic organisms encountered in this study are described in the following pages.

EXPERIMENTAL METHODS

Detection of pectolytic organisms. A sodium polypectate gel medium was used for the detection of pectolytic organisms. The medium, first described by Baier and Manchester *(2)*, was modified as follows for use in these studies:

Sodium polypectate (Exchange)	60.0 g.
Bacto peptone (Difco)	5.0 g.
Bacto beef extract (Difco)	3.0 g.
Calgon (food grade)	2.5 g.
Distilled water	1,000 ml.

(When it was desired to control mold growth, 1.0 g. sodium propionate was added to the mixture of ingredients.)

This medium was prepared in 500-ml. quantities because of the capacity of the available equipment. The dry ingredients were added to 150 ml. of water in a laboratory blender. The mixing was started. Then 350 ml. of boiling water were added. Blending was continued until a smooth, creamy mixture resulted (generally about 2 or 3 minutes). Hydrochloric acid or sodium hydroxide was used to adjust the pH as desired. To favor the growth of molds and yeasts the medium was adjusted to an initial pH value of 4.0 to 4.5, whereas it was used at near neutrality for bacteria. Then the medium was put into tubes or flasks and autoclaved at 15 p.s.i.g. for 15 minutes. This polypectate gel medium when solidified may be remelted for use like agar except that autoclaving is required. However, this practice is not recommended because reautoclaving causes excessive losses from frothing and boiling over. Furthermore, the reautoclaved medium becomes appreciably darkened which indicates heat damage.

The pectate medium was used for collection of qualitative and quantitative data. Brines were tested qualitatively by immersing sterile swabs in the brine samples and

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then smearing the swabs across the surface of the gel contained in petri diches. Samples of small cucumbers, their leaves, and blossoms were collected aseptically and smeared across the surface and then left on the surface of the gel during incubation. The presence of pectolytic organisms was indicated by softening of the polypectate gel under and around the colonies that developed on the medium incubated at room temperature for 3 to 14 days.

Quantitative data were more difficult to obtain. While the gel formed is heat reversible, as noted above, it is not as fluid as an agar medium. Therefore, a very careful mixing of the inoculum and gel was required when plates were poured for subsequent counting. Further difficulty was encountered when the plates were ready for counting. If liquefaction occurred there was a general coalescing of colonies so that it was quite diffcult to determine which one produced the softening. Constant observation of the plates during incubation, which might have overcome this objection was not practical for obvious reasons. Therefore, another, more adaptable method was sought.

The quantitative method finally adopted involved the familiar "estimation by dilution." Liquid samples were diluted in the usual manner. Solid samples were weighed, suspended in sterile distilled water and shaken mechanically for 10 minutes (Vaughn, *22*). Then 1-ml. portions of appropriate dilutions of either type of sample were pipetted into a tube containing about 5 ml. of the polypectate medium which had been solidified on the slant. The first manifestation of the presence of pectolytic organisms was a weakening of the gel in the slant. Longer incubation resulted in complete liquefaction of the slant and usually a portion or all of the gel remaining in the tube. It was assumed that at least one pectolytic microorganism or its spore was present in the last dilution of the sample which caused liquefaction of the gel. (The accuracy of this method can be increased by using the "most probable number" technique described by Prescott, Winslow, and McCrady, *18*).

Collection and treatment of samples. All field samples were collected under aseptic conditions and tested at the point of origin where possible. The soil samples, some brines, and tank shavings were collected in sterile bottles and returned to the laboratory for examination. All of the brines were refrigerated in transit. In addition four large groups of spices were examined in the laboratory. One group was obtained in retail channels; the other three were obtained directly from the spice industry. The latter samples were collected under aseptic conditions by qualified representatives of the spice industry and shipped to Davis.

Isolation and purification of cultures. Colonies of bacteria, molds, and yeasts presumed to liquefy the sodium polypectate gel medium were picked and immediately streaked on nutrient agar that contained 6% sodium chloride. Pure cultures of the various organisms were obtained by restreaking in the conventional manner on this medium as required. The salted medium was used to eliminate cultures that could not tolerate 6% sodium chloride on first contact *in vitro*. This preliminary screening was desirable because the microorganisms capable of softening fermenting cucumbers might have to be able to tolerate even higher concentrations of salt in brined fresh cucumbers.

Purified, pectolytic, salt-tolerant cultures finally were separated by serial passage through the polypectate medium; the same medium plus 6% salt (the medium then is fluid because of the added salt); and then through the unsalted pectate medium. After this final screening, 75 cultures were found to tolerate at least 6% salt and to be able to liquefy the pectate gel medium within 2 days' incubation at 35°C.(95°F.). These isolates were identified by conventional methods.

Other methods. Total titratable acidity was determined according to the method of the Association of Official Agricultural Chemists *(1)* and results were calculated as grams of lactic acid per 100 ml. of brine. Salt was determined by hydrometry or by titration *(1)*. All pH values were determined with the glass electrode. The relative firmness of the cucumbers was determined with the pressure tester *(8, 10, 14)*.

Other pertinent methods will be described in connection with individual experiments.

RESULTS

Sources of aerobic, pectolytic organisms. The widespread occurrence of aerobic, pectolytic organisms is shown by the data presented in Tables 1 and 2. No single source was found which might serve as a focal point for contamination of fermenting "salt stock" cucumbers with softening organisms. Some samples of spices, particularly the

TABLE 1

Incidence of aerobic pectolytic organisms from various sources associated with "salt stock" cucumbers

Source	Total number of samples	Number[1] positive	Percent positive
Soils	42	32	76
Cucumbers from fields	4	2	50
Cucumber blossoms	68	57	83
Cucumber leaves	8	8	100
Fermentation tank shavings	5	2	40
Water	5	0	0
Salt	2	0	0
Fermenting brines	66	59	89.5
Fermented storage brines	133	7	5.2

[1] As determined by visual softening of the polypectate medium.

TABLE 2

Incidence of pectolytic organisms in various spices

Whole spices		Ground spices	
Kind	Count/gm.[1]	Kind	Count/gm.[1]
Allspice	10,000	Allspice	1,000,000
Bay leaves	100	Black pepper	100,000
Black pepper	100,000	Cardamon	1,000
Cardamon	10	Cayenne	10,000
Caraway	1,000	Cumin	1,000,000
Cassia buds	10	Curry powder	1,000,000
Cayenne pepper	10,000	Cinnamon	1,000
Cinnamon, Batavia	10	Clove	0
Clove	100	Ginger	10,000
Coriander	1,000	Mace	1,000
Dill seed	10,000	Marjoram	0
Fennel seed	1,000	Mustard	1,000
Ginger	10	Nutmeg	10,000
Mace	1,000	Paprika	100,000
Mustard seed	0	Pumpkin pie spice	10,000
Pepperone rosso schiacciato	10,000,000	Poultry seasoning	1,000,000
Pickling spice (sterile)	0	Sage	100,000
Pickling spice (not sterile)	1,000,000	Savory	10,000
Red pepper	100	Thyme	10,000
		Turmeric	100,000

[1] Quantitative results based on the dilution method described above.

ground samples, contained rather large populations of pectolytic organisms. These results confirm that softening organisms actually are present on spices, as had been guessed earlier *(7)*. However, spices are not used in the preparation of "salt stock" cucumbers so it is obvious they are not involved in any way in the major outbreaks of softening which are of concern during the pickle salting seasons. These pectolytic populations were composed predominantly of bacteria even though, as noted above, an attempt was made to favor the growth of molds and yeasts by using an acidified medium.

Identification of the pectolytic cultures. The pure cultures of pectolytic, salt-tolerant microorganisms obtained from the survey included 68 bacteria and 7 molds. The bacteria, all members of the genus *Bacillus*, were catalase-positive, mesophilic, aerobic, sporulating, gram-positive, or gram-variable rods which acidified various carbohydrates

without visible gas production. These isolates, identified by the methods of Smith, Gordon, and Clark *(20, 21)* represented four species, as shown in Table 3. These included 43 cultures of *Bacillus subtilis*, 2 cultures of *B. subtilus* var. *aterrimus*, 21 cultures of *B. pumilus*, and 2 cultures of *B. laterosporus*. It is interesting to note that the two predominating species isolated in this survey, *B. subtilis* and *B. pumilus*, were the only pectolytic species found in a previous study reported by Nortje and Vaughn *(15)*.

Seven cultures[*] of *Fungi Imperfecti* included 3 isolates of *Aspergillus*, 3 isolates of *Fusarium*, and 1 isolate of *Penicillium*. Although the acidified polypectate medium did support their growth and they softened it, these cultures have been incorporated in a separate study to determine a more suitable medium for detection of pectolytic fungi. This latter study was thought to be required because a predominant proportion of the colonies that developed on the acidified medium were pectolytic bacteria. It was determined that as the bacteria developed there was a marked increase in the pH value of the acidified medium to 6.5 or higher which, perhaps, could interfere with the growth of fungi. Since the species of *Bacillus* described herein are proteolytic, it is assumed that the observed increases in the pH values of the acidified medium are caused by liberation of ammonia and perhaps carbon dioxide from the beef extract and peptone contained in the medium. The products of degradation of the polypectate also might be involved in the changes in pH values noted.

The possible relationships of pH values and softening. As already stressed, invariably, species of *Bacillus* were the predominating pectolytic organisms recovered from brines of the softened cucumbers encountered in this study. This circumstantial evidence indicated that perhaps bacteria did produce the enzymes that caused the softening. Therefore, because pH is one factor known to have a marked effect on the activity of the pectinolytic enzymes produced by bacteria *(12, 15, 26)*, considerable attention was given to the possible relationships of pH values and softening.

The pH values of fresh cucumbers. Five sizes of cucumbers generally are used in the pickling industry. Adequate samples of these different sizes were collected and tested within 12 hours after harvest. Approximately 1½ pints of homogenate were prepared with each size of cucumber by comminuting in an electric blender. The samples homogenized included whole cucumbers, their skins (the outer 1/16 to ¼ inch or 1.56 to 3.12 mm. sections of the cucumbers), and heart sections (center portion without skins) as well as top, bottom, blossom and stem-end halves; all with skins attached. The pH ranges of the different samples were as follows: whole cucumbers—5.7 to 6.1; skin—6.1 to 6.3; heart—5.8 to 5.9; blossom half—5.7 to 6.1; stem half—5.7 to 6.2; top half—5.8 to 6.0; and bottom half—5.8 to 6.1.

It is apparent that the freshly harvested cucumbers have pH values within the nearly neutral range where the pectinolytic enzymes of the bacilli described above would be active. (See Nortje and Vaughn, *15*.) Yet, since it is well known that the pH values of fermenting brines decrease rapidly *(4, 5, 6, 9)*, it is unlikely that the bacilli would be able to elaborate softening enzymes under normal conditions of fermentation *(15)*. If, on the other hand, the fermentation was slow to start, or was arrested, one might expect the bacilli to predominate the fermentation and then soften the cucumbers, particularly if they could decompose lactic acid.

The utilization of lactic acid. However, there is reasonable cause for conjecture concerning the ability of these bacteria to decompose lactic acid. It is known that the bacilli associated with the softening of cucumbers are not highly tolerant to lactic acid *(15)*. Furthermore, although earlier workers have reported utilization of lactic acid by different species of *Bacillus (11, 25)* study of this characteristic was not included in the most recent detailed taxonomic investigations of these bacteria *(19, 20, 21)*. Therefore, the ability of all isolates to decompose lactic acid was investigated.

Two methods were employed for testing utilization of lactic acid. Bacto yeast nitrogen base was used as the basal medium in both cases. In the first method, 0.5% calcium lactate was added to the Bacto nitrogen base prepared with washed agar. This

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TABLE 3

Characteristics of the bacteria isolated from different field samples

Character[1]	Group 1 43 cultures	Group 2 2 cultures	Group 3 21 cultures	Group 4 2 cultures
	Number of cultures with typical characters			
Cell diameter less than 0.9 μ	43	2	21	2
Sporangia swollen	0	0	0	2
Central or subterminal spores	43	2	21	0
Terminal spores	0	0	0	2
Growth at pH 6.0 or less	43	2	21	2
Gelatin hydrolysis	43	2	21	2
Starch hydrolysis	43	2	0	0
Black pigment in carbohydrate medium	0	2	0	0
Black pigment in tyrosine medium	0	0	0	0
Acetoin from glucose	43	2	21	0
Nitrate reduction	43	2	0	2
Species allocation	*B. subtilis*	*B. subtilis* var. *aterrimus*	*B. pumilus*	B. laterosporus

[1]According to the methods of Smith, Gordon, and Clark *(20, 21)*.

medium, adjusted to pH 6.5 was tubed, sterilized, slanted, and inoculated with the various cultures. The control medium for this method consisted of the nitrogen base medium without added lactate. The inoculated lactate and control media were incubated at 35°C.(95°F.) for one week before examination.

The second method of testing used the conventional auxanogram method. The nitrogen base with added washed agar was sterilized, cooled, poured into petri dishes, one dish for each culture, and inoculated before the medium solidified. Lactate utilization was then tested by placing a small portion of sterile calcium lactate on the surface of the solidified agar near the inner rim of the plate. On the opposite side of the dish a small amount of glucose was added as a control to insure that growth could occur on the medium. The inoculated plates were then incubated at 35°C.(95°F.) for one week before examination. Lactate and glucose utilization was considered to be positive if colonies developed around the points of application of the substrate and not in the other areas of the agar.

All of the isolates grew in the presence of lactate, regardless of the method for testing, whereas the control media without substrate did not support the growth of any of the cultures. Therefore, it was concluded that the organisms could utilize lactate in a nearly neutral environment. Utilization of lactate acid in more acid environments also was tested.

The effect of pH on softening of cucumbers by proliferating cultures. All of the evidence presented above as well as that accumulated in a previous study *(15)* indicated that the pH values of the brines at the start of the fermentation would have a marked effect on the ability of the bacilli to cause softening of the cucumbers. Therefore, experiments to test this effect were made with growing cultures of the bacilli.

Preparation of sterile cucumbers in brine presented a problem. Because the usual methods of sterilization by autoclaving or intermittent steaming caused severe softening, lower temperatures were used. It was found that intermittent heating at 71.1°C. (160°F.) for 30 minutes on three or more successive days did not markedly reduce the original firmness of the cucumbers. However, at the higher pH values (5.5 to 6.5) in particular, sterilization was not always effective. Therefore, a more reliable method for sterilization was sought.

A number of exploratory experiments indicated that the most promising means for sterilization of the brined cucumbers would involve the use of penicillin and mild heat. Pure cultures would grow in the brined cucumbers so sterilized because the residual antibiotic could be neutralized.

Sterilization of the cucumbers was fulfilled by first autoclaving 250 ml. of brine; (0.1 *M* lactate-phosphate buffer with 6% sodium chloride and with or without 25 ml. of

fresh cucumber homogenate) at the desired pH in 500-ml. cotton-plugged wide mouth bottles. Then, after cooling, penicillin was added at a concentration of 2,000 units per ml. of brine, three fresh or desalted cucumbers were submerged in the brine, and the bottles and contents were incubated at 35°C.(95°F.) for 3 days. Then the treated cucumbers were heated at a brine temperature of 71.1°C.(160°F.) for 30 minutes and incubated for 3 more days before penicillinase was added. After additional incubation to test for sterility, the brines were inoculated with pure cultures and incubated at 35°C.(95°F.) for varying lengths of time to test the ability of the bacteria to soften the cucumbers.[4]

It was possible with this method of sterilization to demonstrate that cultures of the bacilli selected at random would grow and soften cucumbers in brines with rather low initial pH values (Table 4). When softening occurred, it was accompanied by an

TABLE 4

The effect of pH on ability of growing cultures of bacillus to soften brined cucumbers

Culture	Final pH of brine	Cucumber firmness, P. S. I.[1]	
		Final (average)[2]	Percent reduction
Test No. 1			
Control[3]	4.0	11.5	0
B. subtilis, No. 95	4.0	11.6	0
B. subtilis var. *aterrimus*, No. 15B	4.0	11.5	0
B. pumilus, No. 15A2	4.0	11.6	0
B. laterosporus, No. 34	4.0	11.4	0
Test No. 2			
Control[3]	4.4	11.6	0
B. subtilis, No. 95	6.6	3.6	68.9
B. subtilis var. *aterrimus*, No. 15B	7.0	0.08	99.3
B. pumilus, No. 15A2[4]	4.4	11.6	0
B. laterosporus, No. 34	6.4	4.2	63.8
Test No. 3			
Control[3]	4.7	10.5	0
B. subtilis, No. 95	7.5	0	100
B. pumilus, No. 15A2	7.1	0.5	95.2

***Conditions:** Each flask contained 3 cucumbers (submerged in 250 ml. of previously autoclaved, buffered brine with 6.0% NaCl) sterilized with mild heat and penicillin, incubated at 35°C.(95°F.) for 17 to 21 days.*

[1] Fruit pressure tester (*8, 10, 14*).

[2] Firmness variable from blossom end to stem end of cucumber so three tests were made; at stem end, at blossom end, and mid point, respectively, for each cucumber. Final firmness an average of all tests for each lot.

[3] Uninoculated but incubated as above. Firmness did not change during incubation.

[4] The limiting pH value for growth of this species is about 4.5.

increase in the pH of the brine if the initial value was significantly acid. Softening was not observed unless the final pH of the brine was above 5.5 even though the cultures had grown before termination of the experiments. These results, with growing cultures, tend to substantiate earlier observations concerning the effect of pH on elaboration and activity of pectolytic enzymes by similar bacilli (*15*). Nonetheless, some credence must be attached to the claim that species of *Bacillus* can cause softening of cucumbers. However, the conditions must be carefully stipulated:

1. The bacilli must predominate the microbial populations of the cucumbers and their brines.

[4] Additional experience has shown that penicillin-resistant, pectinolytic microorganisms sometimes survive. This difficulty has been overcome by the simultaneous use of penicillin and streptomycin.

2. The pH of the brined cucumbers must be in the desirable range—probably pH 5.5 or above.
3. The desirable lactic acid fermentation must be retarded or arrested in some manner so that the pH values of the brined cucumbers remain relatively high for several days. (See Fabian and Bryan, *4*).

DISCUSSION

It might be concluded, on the basis of the results presented above, that the species of *Bacillus* isolated and identified in this study caused all of the commercial spoilage observed by the authors during the 1952 salting season. The pectolytic populations of all samples were predominately bacilli. Only pectolytic bacilli were recovered from the brines of softened cucumbers. Sterile cucumbers in 6% salt brines became soft when inoculated with representative pure cultures of these bacilli if the pH was in a range suitable for their growth and elaboration of their pectolytic enzyme systems. This evidence, substantial by itself, is strengthened by observations taken in the field. Softening became apparent in the commercial plant almost at the beginning of the cucumber harvest the first of July, 1952, and remained acute for several weeks. It was evident from total acid titrations and pH determinations that the desirable lactic acid fermentation was slow to start. Manipulation of the salt concentration in the brines had no effect in arresting the spoilage. Slippery, soft, and mushy pickles occurred until massive inoculations of fresh, normal, actively fermenting brines were added to each new tank of cucumbers. As a result, the lactic acid bacteria received such an advantage that the spoilage types were not able to develop significantly. In any event, no further spoilage occurred during that season and none was observed in 1953.

In all fairness, however, it is to be emphasized that the conclusions reached here should be subjected to further investigation. It may be that fungi or even other bacteria actually played a more important role in the softening spoilage than the evidence presented in this paper would indicate.

Pectolytic fungi including representatives of the genera *Fusarium*, *Aspergillus*, and *Penicillium* were recovered from some of the field samples examined in this study. If field sanitation is a factor in softening spoilage, then it must be determined whether pectolytic bacteria or fungi predominate the populations in such samples. It must also be determined whether either or both groups of microorganisms cause significant build up of pectolytic enzymes in field cucumbers, their blossoms, and leaves or whether the claimed enzyme system *(3)* is intrinsic to the cucumbers. Too, it must be remembered that other bacteria may be involved in softening spoilage. The "soft rot" bacteria and the anaerobic, retting bacterium *Clostridium felsineum* are known to decompose pectinous materials *(12, 17, 26)*. Unpublished, and as yet incomplete, investigations indicate that bacteria of the *Bacillus polymyxa-Bacillus macerans* group could cause softening of cucumbers. Representative cultures of these bacteria cause rapid softening and gas formation in the polypectate gel medium. They also ferment purified pectic acid and α-D-galacturonic acid as energy sources; both with copious gas production. *In vitro* studies already reported indicate that these bacteria can survive and grow in environments having pH values as low as 3.8 *(23, 24)*. The limiting factor would appear to be salt tolerance

which has been reported as less than 10% for the most resistant, trained cultures of these bacteria *(21)*.

SUMMARY

An attempt was made to determine the sources and types of microorganisms which might be associated with the softening of salt stock cucumbers. Soils in which cucumbers were grown, cucumbers, their blossoms and leaves, shavings from fermentation tanks, water, salt, brines, and spices were tested for the presence of organisms that caused softening of a polypectate gel medium. No single source was found which might serve as a focal point for contamination of fermenting salt stock cucumbers with softening microorganisms. Rather, each source investigated might serve as a source of contaminating organisms except spices which are not used in this fermentation of cucumbers.

The predominating microorganisms found were aerobic, sporeforming bacteria of the genus *Bacillus*. There were 43 of these isolates that belonged to the species *Bacillus subtilis*; 2 cultures identified as the species *B. subtilis* var. *atterimus*; 21 as cultures of *B. pumilus*; and 2 cultures representative of the species *B. laterosporus*. In addition 7 isolates of molds were isolated and purified. These included 3 cultures of *Fusarium solani*, 3 cultures of *Aspergillus* and 1 culture of *Penicillium*. All of the purified cultures (bacteria and fungi) softened the polypectate gel and grew in the presence of at least 6% salt. The species of *Bacillus* predominated under the conditions of these experiments.

The bacilli were able to utilize lactic acid if its concentration was not inhibitory. It was concluded, therefore, that the species of *Bacillus* might cause softening of salt stock cucumbers if:

1. The bacilli predominated the microbial populations of the cucumbers and their brines.
2. The pH of the brined cucumbers was in the desirable range for softening—probably pH 5.5 or above. It was found that the bacilli could raise the pH of the brines unless the initial values were inhibitory.
3. The desirable lactic acid fermentation was retarded or arrested in some manner so that the pH values of the brined cucumbers remained relatively high for several days. (See Fabian and Bryan, *4*.)

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USE OF FINELY EMULSIFIED FATS FOR THE DETERMINATION OF LIPASE ACTIVITY[a]

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Various titrimetric and manometric methods have been proposed for the quantitative measurements of enzymatic activity of pancreatic and other lipases. Since these procedures have been recently reviewed by Bradshaw *(4)* and by Ammon and Jaarma *(1)*, the details of the methods will not be discussed in this paper, except to call attention to certain features which adversely affect the precision and accuracy of the determination. Thus, it appears that such procedures include one or more of the following undesirable practices: the use of poorly dispersed, unstable fat emulsions which prevents the maximum interaction of the enzyme-substrate complex; the inadequate control of the pH during the course of reaction which introduces important variables affecting the precision of the results; and the resort to solvent extraction of the fatty acids from the digest mixture which permits manipulative errors and time-consuming labors.

The present investigation was undertaken to develop a more sensitive and convenient method for determining the digestibility of fat by pancreatic and other lipases. A technique for dispersing lipid substrates in a finely divided and stable emulsion was employed, using a soy phospholipid preparation as the emulsifying agent and carrying out the emulsification in a high-pressure dairy homogenizer (Cherry-Burrell Junior Viscolizer). The details for preparing the emulsion were the same as those previously described for studies on the bacterial hydrolysis of fats by the present authors *(7)*. Emulsions so prepared show no oil separation during prolonged storage, and the uniform globule size of less than 0.5 micron makes available an enormous surface for lipase action.

Since the fat-splitting action of lipase is greatly affected by changes in pH, it seemed advisable to maintain the optimum pH level by continually adding dilute alkali to the digestion mixture during reaction, as was done by Koch and Duellman *(9)*, and Bullock *(5)*. The addition of an indicator to the digestion mixture was found in the present work to be unsatisfactory in controlling the pH, partly because of the interfering turbidity of the digest. Also, Bamann and Schmeller *(3)* have indicated the possibility of significant errors inherent in the use of indicators in the esterase determination because of their inhibitory effect on the enzyme-substrate system. Therefore, the pH was maintained in this study by the use of the glass electrode as described by Glick *(6)* for the determination of choline esterase. With this procedure, the functions of both pH control and titra-

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tion of free fatty acids were performed simultaneously and with increased precision and convenience.

EXPERIMENTAL

Stock emulsions. Oil-in-water emulsions were prepared as described previously *(7)* in oil concentrations of approximately 20 g. per 100 ml. The emulsifying agent employed at a level of 2 g. per 100 ml. was a commercial soy phospholipid preparation known as Astec 4135.[b] After homogenization the fat content of each batch of emulsion was determined and the emulsions were diluted to a standardized concentration of 20%. This standardization of the fat concentration of each batch of emulsion was necessary owing to the variable evaporation of water during homogenization.

Determination of fat in emulsions containing phospholipids. The presence of the phospholipid emulsifier, Astec, in the emulsion precluded the determination of triglyceride concentration by direct saponification of the emulsion. However, it was feasible to determine the triglyceride content by an indirect analysis in which the volume of alkali consumed in saponification of the phospholipid was subtracted from the total alkali used in saponifying the emulsion. The volume of alkali consumed by saponification of the phospholipid in the emulsion was calculated from the following values obtained by analysis of the phospholipid: (a) saponification value and (b) per cent phosphorus. Astec was the only source of phosphorus in these emulsions since the phosphorus content of the fats and oils studied was found, on analysis, to be absent or insignificant. Therefore, it was possible to calculate the phospholipid content in such a mixture of phospholipid and triglycerides by determining the quantity of phosphorus present; and the volume of alkali consumed in saponifying the phospholipid fraction in the emulsion could then be computed, using the saponification value of the phospholipid.

Phosphorus was determined by a modification of the method of Kitson and Mellon *(8)*.

Saponification of anhydrous fats and phospholipids was performed by the A.O.A.C. method *(2)*, using a reflux period of 2 hours instead of the suggested 30-minute period. For saponification of emulsions, 20 ml. of emulsion was substituted for the fat.

Determination of lipase activity. A standardized procedure for determining lipase activity has been developed and, unless otherwise stated, was conducted in the following manner: Into a 250-ml. beaker, securely anchored in a 35°C. (± 0.01°) water bath, were added 10 ml. of 20% fat emulsion, 5 ml. of phosphate buffer, and 30 ml. of water. The primary potassium phosphate buffer was 0.1 M, pH 8.2. Glass and calomel electrodes, attached to a Beckman Model H2 pH meter, and a motor-driven glass stirrer were immersed in the buffered emulsion which was then brought to bath temperature.

Two such titration setups were used in this study (Figure 1). The use of a sleeve-type calomel electrode for pH measurement is recommended, since the wick-type has a high resistance in oily emulsions which causes drifting meter readings. The pH was readjusted to 8.2 with dilute NaOH and then there was rapidly added to the reaction mixture 5 ml. of a saline suspension which contained 5 to 50 mg. of the lipase preparation, depending upon the potency of the enzyme. If a dried enzyme preparation is to be assayed, water rather than saline, may be used for suspending the sample, provided the suspension is added without delay to the buffered emulsion. The reaction was carried out for one hour at 35°C. with gentle stirring of the mixture, while aqueous 0.1 N NaOH was added at 5-minute intervals during the first half hour, and at 10-minute intervals during the second half hour in order to maintain the pH at the initial level.

One hour was selected as the digestion period because it yielded titers which were highly reproducible and because it afforded enough time for lipase preparations of low activity to develop a suitable degree of hydrolysis. The total volume of standard alkali used during the reaction was recorded. No correction was made for the acidity contributed by partial hydrolysis of the emulsifying agent, Astec, since the small amount of agent in the emulsion, and its relatively high resistance to lipase action made this a negligible quantity. For the experimental controls the same procedure was followed, substituting for the active enzyme a suspension of the lipase heated in boiling water for 20 minutes. This control titer is subtracted from the value obtained with the active enzyme and the difference is expressed in the experimental data as ml. of 0.1 N NaOH. Data presented in the tables and graphs represent averages of duplicate or triplicate assays.

[b] Associated Concentrates, Inc., 57-01 32nd Ave., Woodside, N. Y.

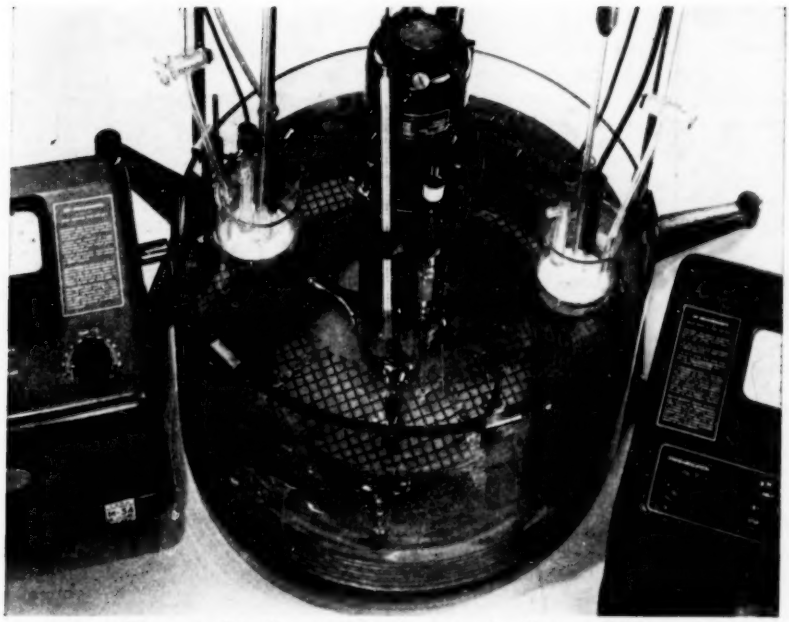

Figure 1. Titration assembly for measuring lipase activity.

RESULTS

Data in Figure 2 demonstrate the effect of pH on the activity of pancreatic lipase. Hydrolysis was studied in emulsions buffered with KH_2PO_4, NaOH mixtures, at pH levels of 7.5, 7.8, 8.0, 8.2, 8.5, 8.7, and 9.0. Maximum lipolysis occurred at pH 8.2.

The influence of temperature upon lipolytic activity of pancreatin is shown in Figure 3. A temperature optimum of 35°C. was demonstrated for different pancreatic lipase preparations.

When lipolytic activity was measured at 60 minutes against the concentration of enzyme, the linearity of response depended upon the amount of lipase reacting. Thus, at low enzyme levels of 2 to 10 mg. of steapsin a straight-line relationship existed between the activity plotted logarithmically and the lipase concentration, whereas at higher enzyme levels of 10 to 50 mg. the plotted relationship resulted in a non-linear curve (Figure 4).

Data represented in Figure 5 show the effect of substrate concentration upon lipolytic activity measured at 60 minutes. Although the curve shown is for butter fat, similar results were obtained when corn, soy, and olive oils were used as substrates. Lipolysis increased as the substrate concentration was increased and maximum activity was not attained with the concentrations studied. Even when a similar range of butter fat levels was exposed to other concentrations of lipase (10 mg. and 50 mg.), maximum lipolysis was not reached.

Reaction rate curves in Figures 6 and 7 show the influence of enzyme levels and fat concentrations, respectively, on the rate of fat hydrolysis. It is apparent (Figure 6) that at a constant fat level the velocity of lipolysis increased with increasing enzyme concentrations, and it can be shown that the reaction rate, k, is directly proportional to the enzyme level. The rate constants, k, were derived from the slopes of the straight line portion of rate curves plotted as $x = kt$. At a constant enzyme level a similar increase in velocity with increasing fat substrate concentrations can be noted (Figure 7). While data were plotted according to typical zero, first, and second order equations in an attempt to obtain straight line curves, linear fits were not satisfied over

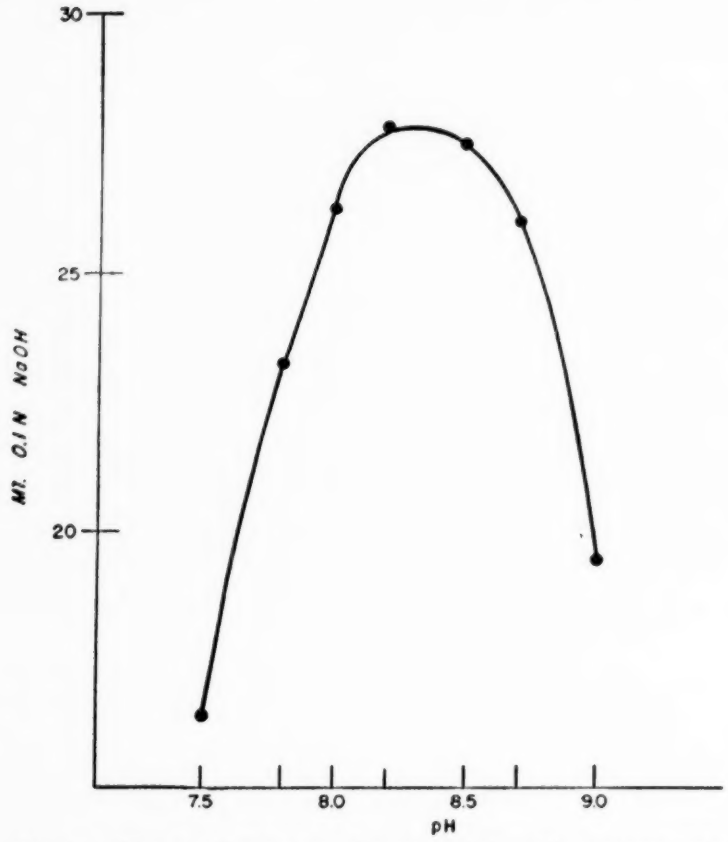

Figure 2. Effect of pH on activity of pancreatic lipase. Digest mixture contained 10 ml. of 20% butterfat emulsion, 5 ml. 0.1 M KH_2PO_4, NaOH buffer, 30 ml. dist. water, 50 mg. No. 11 lipase suspended in 5 ml. water. Reacted 1 hour at 35°C.

the entire time period by any of the functions tested. Although these kinetic results are not strictly conclusive, nevertheless the data in this study indicate that when fat substrates are in a finely divided state the enzyme kinetics approach that of homogeneous solutions investigated by Schønheyder and Volqvartz *(10, 11, 12)*.

Action of lipase upon several substrates was studied by exposing emulsions of corn, olive, and soy oils, in addition to butter fat, to pancreatic lipase. An added improvement in this experiment was the incorporation of activators (calcium chloride, albumin, and sodium taurocholate) to the reaction mixture. Results obtained with these different fats (Table 1) demonstrated that these fats of quite different molecular composition can be split to the same extent by enzymatic action if the fat emulsions are equally well dispersed. It may be noted that the same rate of hydrolysis held for these different fats.

Typical values demonstrating the reproducibility of this method with replicate samples are presented in Table 2. A degree of precision could be attained in which the standard deviation from the mean was less than 0.25 ml. of 0.1 N NaOH, within a reasonable range of titration values.

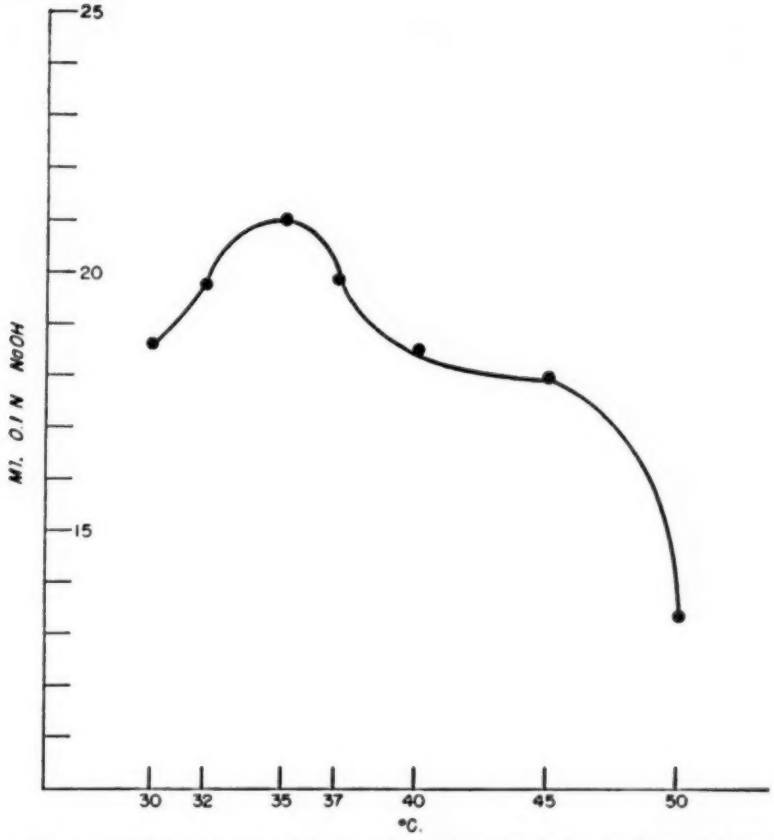

Figure 3. Effect of temperature on activity of pancreatic lipase. Digest mixture contained 10 ml. of 20% butterfat emulsion, 5 ml. 0.1 M KH_2PO_4, NaOH buffer pH 8.2, 30 ml. dist. water, 25 mg. No. 11 lipase suspended in 5 ml. water. Reacted 1 hour at 35°C.

TABLE 1

Action of lipase on different fat substrates

Substrate	Enzyme hydrolysis, ml. 0.1 N NaOH (x)	Hydrolysis by saponification, ml. 0.1 N NaOH (a)	Per cent hydrolysis, x/a × 100
Butterfat	33.65	79.20	42.48
Olive oil	28.60	69.20	41.32
Soybean oil	25.65	68.40	37.50
Corn oil	29.80	69.40	42.93

Digest mixture:
10 ml. of 20% fat emulsion.
5 ml. KH_2PO_4, NaOH buffer, pH 8.2.
5 ml. 2% $CaCl_2$.
5 ml. 4% albumin.
5 ml. 2% sodium taurocholate.
10 ml. water.
10 ml. saline containing 50 mg. No. 11 lipase.
Reacted 1 hour at 35°C.

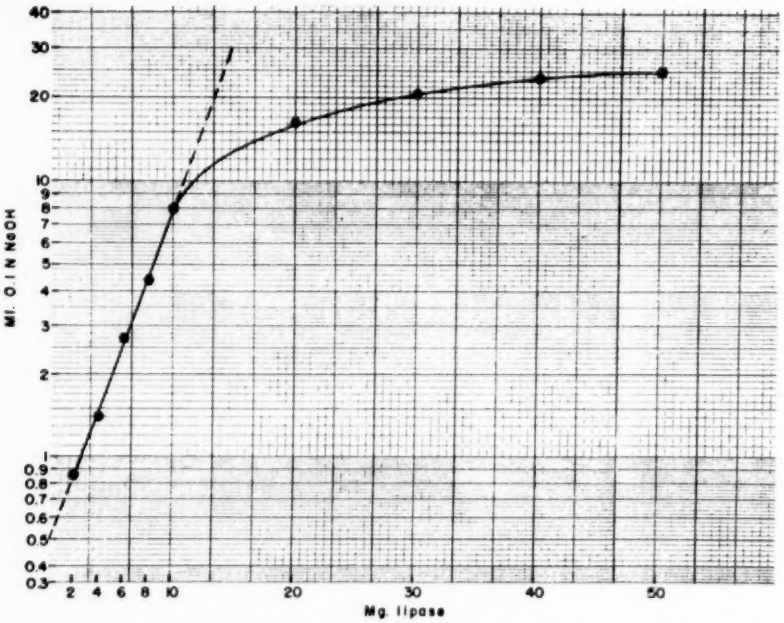

Figure 4. The effect of lipase concentration upon its activity. The digest mixture contained 10 ml. of 20% butterfat emulsion, 5 ml. 0.1 M KH_2PO_4, NaOH buffer pH 8.2, 30 ml. dist. water, 25 mg. No. 11 lipase suspended in 5 ml. water. Reacted 1 hour at 35°C.

Data in Table 3 show the sensitivity of the proposed method compared with the original method of Willstätter, Waldschmidt-Leitz, and Memmen *(13)*, a procedure which has been widely cited in the literature. The Willstätter method, which uses crude emulsions, was performed as described by its authors except that the free fatty acids were titrated potentiometrically rather than with the use of thymolphthalein indicator. For each method the digest mixture contained 2.5 gm. fat, 50 mg. lipase No. 11, and Willstätter's activators, calcium chloride and albumin. Procedural differences such as mode of fat dispersion, composition of buffer, and manner of pH control and titration were as cited for the two methods. The reaction proceeded at 30°C. for one hour.

Considerable variation in activity exists among commercial pancreatic lipases marketed as "Steapsin" or "Pancreatin." Table 4 illustrates the variation in enzyme potency of 10 different preparations representing products from 5 companies. Three powders were weakly lipolytic, one was moderate in strength, and six demonstrated high potency. As might be expected, preparations labelled "Steapsin" appeared to have a uniformly higher activity than "Pancreatin" samples.

To determine whether these differences in potency might be minimized by more careful handling of the enzyme the stability of lipase was investigated. Five-ml. portions containing 20 mg. lipase were withdrawn at intervals from well-stirred suspensions of enzyme held at 30°C. and 4°C. and were reacted with butter fat emulsion in the usual manner. A loss in activity was found to result from allowing pancreatic lipase to stand in aqueous suspension. This rate of loss was diminished by suspending the enzyme in saline and was also decreased by keeping suspensions refrigerated (Table 5).

The use of 50-70% glycerol as a medium for suspending commercial lipase powders did not offer any advantage over either water or saline. The possible advantage of grinding lipase powders in a mortar in order to get a finer and more uniform suspension was tested and this refinement was not found to produce any higher activity

TABLE 2

Reproducibility of lipase determinations

Weight of lipase (mg.)	Titer found	Mean titer	Standard deviation
	Ml. 0.1 N NaOH		
10	7.05 7.12 7.08 7.15	7.10	0.04
20	14.05 14.40 14.22 14.35	14.25	0.16
30	20.82 21.05 21.07 21.05	21.00	0.12
50	26.15 25.88 26.45 26.20	26.17	0.23

Digest mixture:
10 ml. 20% butterfat emulsion.
5 ml. KH_2PO_4, NaOH buffer, pH 8.2.
30 ml. water.
5 ml. aqueous suspension of No. 11 lipase.
Reacted 1 hour at 35°C.

TABLE 3

Evaluation of lipase activity by two different methods

Substrate	Method	Ml. 0.1 N NaOH	Per cent hydrolysis[1]
Olive oil	Willstätter, *et al.*	3.40	4.90
	Proposed	23.71	34.26
Butterfat	Willstätter, *et al.*	13.57	17.13
	Proposed	27.19	34.33

50 mg. No. 11 lipase. 1 hour at 30°C.
[1]Calculated as in Table 1.

TABLE 4

Assay of commercial pancreatic lipase preparations

Preparation	Designation	Company	Ml. 0.1 N
1	Steapsin	A	10.90
3	Pancreatin	B	2.50
4	Pancreatin	C	13.85
5	Steapsin	C	15.65
6	Steapsin	C	13.92
8	Steapsin	D	17.63
9	Steapsin	D	15.40
11	Steapsin	C	15.70
12	Pancreatin	E	2.60
13	Pancreatin	C	4.71

Digest mixture:
10 ml. of 20% butter fat emulsion.
5 ml. KH_2PO_4, NaOH buffer, pH 8.2.
30 ml. water.
5 ml. aqueous suspension 20 mg. lipase.
Reacted 1 hour at 35°C.

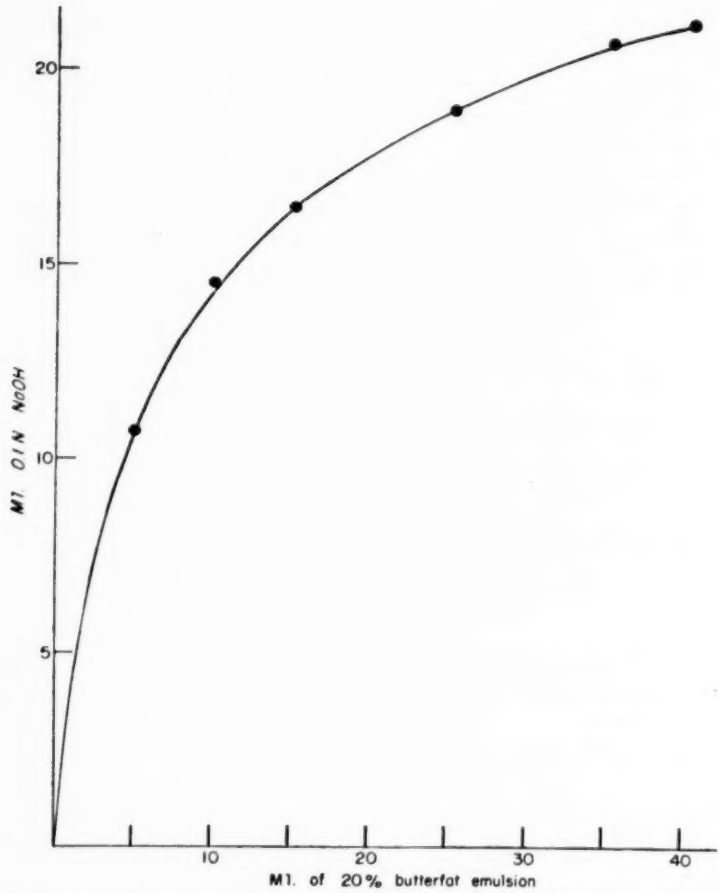

Figure 5. Effect of fat concentration on activity of pancreatic lipase. Digest mixture contained 20% butterfat emulsion, 5 ml. 0.1 M KH_2PO_4, NaOH buffer pH 8.2, 20 mg. No. 11 lipase suspended in 5 ml. water, water to total volume of 50 ml. Reacted 1 hour at 35°C.

among the commercial preparations so treated. However, it was found that some experimental preparations of pancreas powder, which dispersed quite poorly in water, saline, or glycerol, had to be thoroughly ground in a mortar before the suspension could demonstrate any activity. Another factor investigated was the thermal stability of the dry pancreatic lipase when stored at refrigerator and moderately elevated air temperatures. Data in Table 6 demonstrate that lipase, even in the relatively stable powder form, suffers some loss in activity at 37°C. and 55°C., but is unchanged at 4°C. for at least 6 months.

DISCUSSION

The importance of having finely dispersed and stable emulsions for studying enzyme hydrolysis of fats has been clearly demonstrated in this

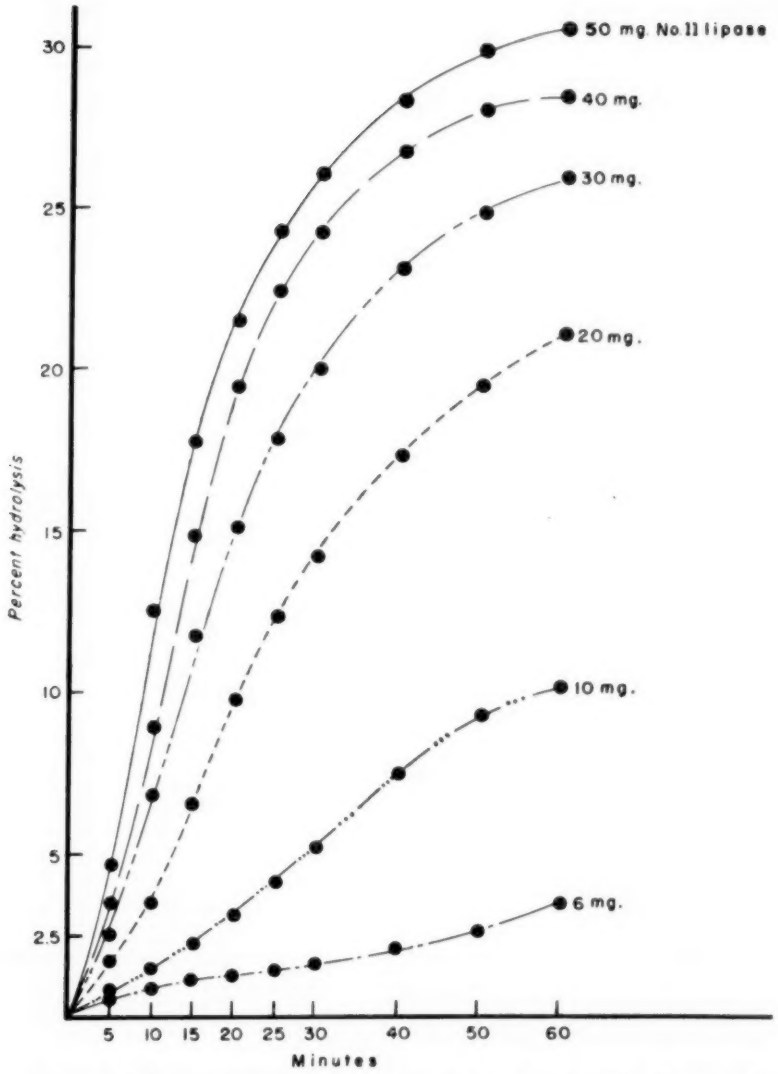

Figure 6. Influence of enzyme concentration on the rate of lipolysis. Digest conditions same as in Figure 4.

study. Fats of widely varying fatty acid composition, when in a state of fine dispersion, were split to the same degree and at the same rate, indicating that susceptibility to hydrolysis is more dependent upon the particle size of the emulsified fat than on the molecular composition of the component glycerides. This observation has also been reported by the present

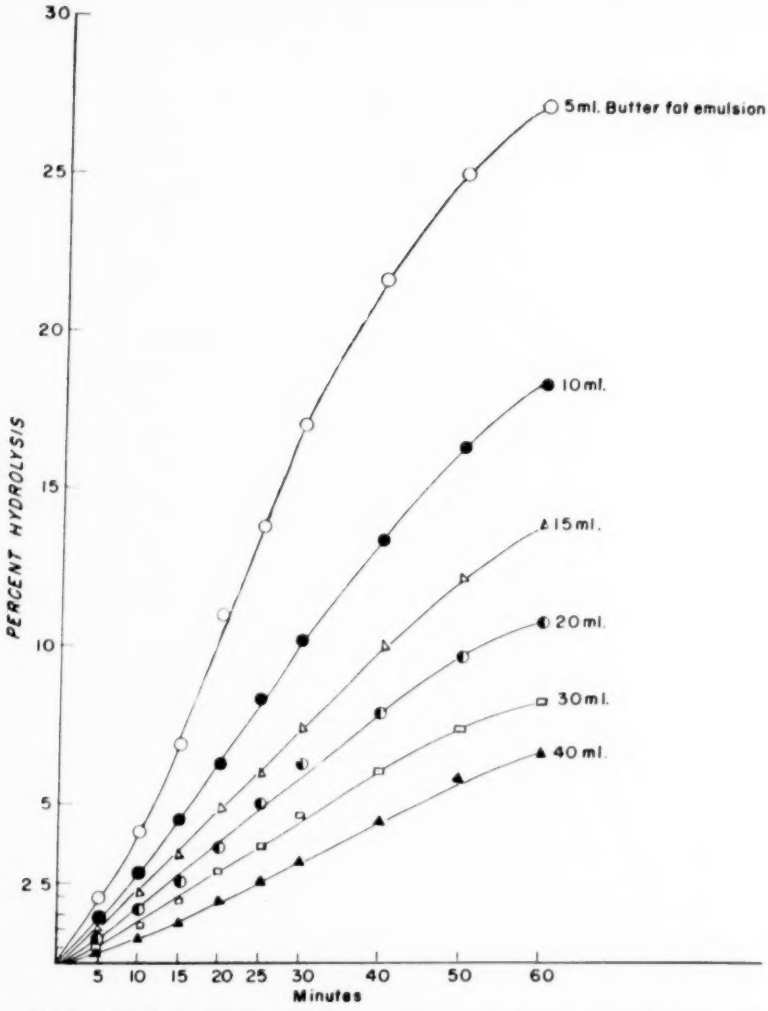

Figure 7. Influence of fat substrate concentration on the rate of lipolysis. Digest conditions same as in Figure 5.

authors in the case of a bacterial lipase acting upon a variety of animal and vegetable fats *(7)*. It is true, however, that some fats are more difficult to emulsify; this was the case with soybean oil in the present study. Soybean oil was not dispersed quite so uniformly as were the other substrates as measured by particle size; this factor was reflected in the slightly lower degree of lipolysis attained by the enzyme acting upon this substrate.

Although activating substances, such as calcium chloride, albumin, and sodium taurocholate were used in only one set of experiments presented

TABLE 5

Stability of lipase suspensions

Age of suspension in hours	Ml. 0.1 N NaOH			
	Aqueous suspension		Saline (0.85%) suspension	
	30°C.	4°C.	30°C.	4°C.
0	13.40	14.20	15.20	15.20
½	12.60	14.08	14.63	15.20
1	11.53	13.00	14.60	15.20
1½	11.05	12.68	14.20	15.11
3	9.00	.....	12.90	15.00
3½	7.55	12.20	.....	14.25
5½	4.80	12.01	12.80	14.00
24	1.82	10.82	6.81	13.99

Digest mixture:
10 ml. of 20% butterfat emulsion.
5 ml. KH_2PO_4, NaOH buffer, pH 8.2.
30 ml. water.
5 ml. aqueous suspension 20 mg. No. 11 lipase.
Reacted 1 hour at 35°C.

TABLE 6

Stability of lipase powder stored at moderately elevated temperatures

Storage temperature	Ml. 0.1 N NaOH		
	Initial	1 month	6 months
4°C.	14.30	14.30	14.30
37°C.	.....	13.70	12.66
55°C.	.....	11.80	8.83

Digest mixture:
10 ml. of 20% butterfat emulsion.
5 ml. KH_2PO_4, NaOH buffer, pH 8.2.
30 ml. water.
5 ml. aqueous suspension 20 mg. No. 11 lipase.
Reacted 1 hour at 35°C.

above, the presence of these agents, either individually or in combination, was found to enhance lipolytic action to some extent. It is interesting that the effect of sodium taurocholate in increasing hydrolysis possibly was not due to its well-known emulsifying ability but to some other mechanism, since the emulsions to which it was added were already in a highly dispersed and stable state.

The observations made concerning the stability of pancreatic lipase indicate that some care should be taken to determine whether or not one is working with a preparation of constant potency. Since pancreatic powders deteriorate in activity upon standing, the use of fresh preparations is strongly indicated; moreover, these powders should always be protected from warm weather temperatures. It is unnecessary to use glycerol as a suspending medium if the powder dissolves readily in water; but it is important, once the powder is put into suspension, that it react with its substrate without delay. If a suspension of lipase is to be used over a period of time, it appears desirable to suspend the preparation in cold saline. These remarks are confined primarily to those enzyme preparations in the dry powder form. For liquid preparations, such as tissue extracts, it may be necessary to employ glycerol or other solvents to extract the enzyme.

SUMMARY AND CONCLUSIONS

An improved procedure for the determination of lipase activity has been developed. By using finely-emulsified fats (globule diameters less than 0.5 μ) as substrates, greater surfaces were provided for enzyme reactions which resulted in higher and more reproducible titers. The optimum pH for enzyme action was maintained throughout the lipolysis by potentiometric titration.

When fats of widely different molecular composition (butterfat, corn, soy, and olive oils) were finely emulsified, the markedly high degree of lipase action obtained was essentially equal for all fats tested.

For pancreatic lipase, optima of pH 8.2 and temperature 35°C. were established.

The degree of lipolysis increased with the concentration of the enzyme, a linear relationship existing at low levels of enzyme when the logarithm of acidity was plotted against the concentration of lipase.

Calculations of rate constants indicated that at the same initial fat level the velocity of lipolysis was directly proportional to the enzyme concentration.

Thermal stability of lipase in the dry state and in aqueous media is discussed.

Considerable variation in enzyme potency was exhibited among different commercial preparations of pancreatic lipase.

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CHEMICAL CHANGES TAKING PLACE IN THE CRUDE LIPIDS DURING THE STORAGE OF FROZEN RAW VEGETABLES[a]

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Recently it was shown that substantial changes had taken place in frozen unblanched peas which were held in storage at 0°F.(−17.8°C.) for extended periods of time *(2)*. These changes were observed in the crude lipid (ether extract) which was extracted from samples of these peas. Crude lipid extracted from the corresponding sample of blanched peas which had been held under the same conditions and for the same period of time and lipid extracted from freshly harvested blanched and unblanched peas showed no such changes. It was concluded that developing rancidity of the lipid matter was one of the primary causes, if not the principal cause of the development of off-flavor in peas so stored. This work has been extended to include other vegetables, and the results are presented herewith.

EXPERIMENTAL PROCEDURE

Preparation of sample. Vegetables and varieties were employed in this study as follows: asparagus, Martha Washington; corn, Golden Cross; lima beans, Fordhook; snap beans, Tendergreen; and spinch, variety unknown. Additional data were collected also on peas, Alderman and Topper varieties. The corn, snap beans, peas, and spinach were grown on the Station farm. The asparagus was obtained from a farm about ten miles from the Station, and the lima beans were brought from a near-by packing plant. In each case, a blanched and a raw (unblanched) sample were prepared.

Asparagus stalks, medium diameter, were cut to 5-inch lengths, discarding the butt. The material was blanched in flowing steam at 210-212°F.(99-100°C.) for 3½ minutes, cooled in cold water, and drained. The asparagus was packed in No. 2 tins, frozen, and stored at 0°F.(−17.8°C.). The corn was mechanically husked, and that to be blanched was placed in flowing steam at 210-212°F.(99-100°C.) and blanched for 7½ minutes prior to cooling in cold water. The kernels from blanched and unblanched material were cut from the cob with mechanized equipment. The lima beans were vined and passed over a sorting belt to remove foreign matter and defects. Those to be heat treated were blanched in boiling water for 2½ minutes, cooled in cold water, drained, and packaged. The unblanched beans were washed in cold water, drained, and packed. The snap beans were snipped and cut into 1-inch lengths. The lot to be heat treated was blanched in boiling water for 2 minutes, cooled in cold water, drained, and packed. The peas were vined, washed, and hand picked to eliminate foreign matter, damaged peas, and skins. Those to be blanched were immersed in boiling water for 60 seconds, cooled, and drained. Blanched and unblanched samples were packed in tub shaped waxed cartons.[b] The spinach leaves were cut off, washed, and drained. The material to be heat treated was blanched in boiling water for 2½ minutes, cooled in very cold water, drained, and packed. The vegetables other than peas and asparagus were packed in 30-pound tins with friction lids prior to freezing and were stored at 0°F.(−17.8°C.).

Extraction of crude lipid. Two hundred grams of the frozen vegetable (150 g. in the case of spinach) were dried in the lyophilizer (2). Lyophilization was considered complete when the pressure in the drying chamber had reached about 5 to 7 μ, which was measured by means of a Pirani gauge. This drying operation took about 2½ days.

[a]Approved for publication as Journal Paper No. 963 by the Director, New York State Agricultural Experiment Station.

[b]Kindly supplied by Lily Tulip Cup Corporation, New York, N. Y.

The drying and dried materials were carefully protected from light. The dried vegetable sample, ground to a powder, was extracted for 24 hours in all-glass Soxhlet equipment using pure, peroxide-free, redistilled, anhydrous ethyl ether as a solvent. Previously *(2)*, a 48-hour extraction time was employed. It was found in the present investigation that the quantity of crude lipid extracted in 48 hours was usually only 0.01 to 0.03% higher than the amount extracted from another sample of the same lot of vegetable in 24 hours, and sometimes the figures obtained for the 24-hour extraction were the same as those for the 48-hour extraction. Within experimental error, therefore, the percentage of crude lipid extracted was about the same for either period of time. However, the peroxide values of that material extracted in 24 hours were a little higher than those obtained on analysis of the material prepared by the 48-hour extraction. It seems, perhaps, that some destruction of peroxide takes place during the longer extraction period. Still shorter extraction periods may result in slightly larger peroxide figures, but they were not tried in the present investigation.

The extraction equipment was fitted with a drying tube so that the contents were protected from moisture during the extraction process, and the equipment was completely protected from light. This precaution was followed throughout all the stages of the work. Tests showed that sunlight is effective in increasing the peroxide values of the crude lipids. This result is in line with that obtained by Lea *(1)*. These data will be presented in the near future in another paper.

Analytical procedure. The ether extract was concentrated under reduced pressure and an aliquot containing 20 mg. of crude lipid was transferred to the 25 mm. × 110 mm. glass stoppered tubes for iodine numbers. For the peroxide and acid numbers Pyrex test tubes 18 mm. × 85 mm. were found to be satisfactory. The ether was removed from each tube by vacuum before weighing. Since almost no water was introduced, this method permitted weighing without further drying. This is of particular value in the determination of the peroxide numbers because it was found, as shown in Table 1, that the

TABLE 1

Loss of peroxide content of extracted crude lipid during storage in a vacuum desiccator over phosphorus pentoxide

Vegetable and sample number	Treatment	Years in frozen storage previous to extraction	Extracted lipid	
			Days of storage	Peroxide values
Asparagus (1)	Raw	7½	0	5.0
			2	0.6
			40	0
Asparagus (2)	Raw	7½	0	5.8
			11	0
Peas	Raw	7½	0	39.9
			6	21.3
			20	7.7
			48	2.1
Snap Beans	Raw	6	0	24.1
			10	2.2
Corn	Blanched	2½	0	8.4
			3	8.0
	Raw	2½	0	45.6
			10	30.1
Lima Beans	Raw	6	0	3.7
			25	0.57
Spinach	Raw	6	0	33.6
			5	8.7

peroxide is lost progressively as the time between preparation of the sample and analysis is lengthened. The error introduced by weighing directly is small, but the error introduced by the loss of peroxide on standing for a period of time can be very large. Check tests showed that this precaution is not necessary for the samples to be used for the determination of acid and iodine numbers. However, the crude lipid samples from each vegetable were analyzed for acid and iodine numbers immediately after evaporation and weighing, and after taking to constant weight over phosphorus pentoxide in the vacuum desiccator. Samples for peroxide values were not taken to constant weight in the desiccator because the figures obtained on analysis after standing for two or more days were worthless.

All weighings were made in a minimum of red light, and all work except the final titrations were done in this light or in the dark, for reasons noted earlier in this paper.

The analytical methods employed for peroxide number, iodine number, and acid number were micromethods, and were fully described in a previous publication *(2)*, with the following modifications: It was found desirable to use only 20 mg. (±10%) as the weight of the sample. Five ml. instead of 1 ml. of distilled water was added to the peroxide test just previous to the addition of starch indicator and titration. Ten ml. of carbon dioxide-free distilled water was added to the samples for acid number determination previous to titration. More satisfactory results were obtained in this fashion. It was found necessary to titrate the prepared samples of spinach lipid to pH 8.2 instead of the usual visual indicator.

Peroxide number, the number of millimoles of peroxide oxygen in 1 kg. of lipid, is calculated as follows:

$$\frac{\text{Ml. of } Na_2S_2O_3 \text{ solution} \times \text{normality factor} \times 5}{\text{Sample weight in grams}}$$

$$\text{Normality factor} = \frac{\text{normality}}{0.01}$$

RESULTS AND DISCUSSION

The results of the chemical examination of the crude lipids of asparagus, corn, lima beans, snap beans, spinach, and the additional work on peas not covered in the earlier publication *(2)* are presented in Table 2.

It is obvious from an inspection of Table 2 that corn, snap beans, and spinach are similar to peas in that high peroxide numbers and high or fairly high acid numbers are found when the crude lipids—extracted from the unblanched, stored vegetables—are analyzed. Similar crude lipid extracted from unblanched asparagus gives no peroxide value except in the case of the very old vegetables (7½ years in storage), but a high acid number; while the crude lipid extracted from raw lima beans gives a low peroxide number and a low acid number. The results indicate that the iodine numbers show considerable variation and are useless to show differences in the condition of the crude lipid matter. This differs from the conclusion reached in the previous paper *(2)*, but the additional work brought to light the results which support this present conclusion. This is true for the Alderman and Topper peas, as well as for the other vegetables. It is further supported by other but unpublished data obtained in this laboratory. The peroxide values for the peas are higher than those reported in 1951 *(2)*. Although it is true that the varieties differ from the one used for the work reported in the earlier paper, it seems that the differences can be explained by the facts that the peas were held in storage for 2½ years longer than those used in the earlier work, and also by the change in technique employed in running this determination.

TABLE 2

Peroxide number, iodine number, and acid number of crude lipid material extracted from vegetables[1]

Vegetable and variety	Years of storage at 0°F.(−17.8°C.)	Treatment	Peroxide number	Iodine number	Acid number
Asparagus					
Martha Washington	2½ yrs.	Unblanched	0	120.9 (117.4-124.7)	86.8 (85.4-88.2)
	2½ yrs.	3½ min. steam blanched	0	85.6 (84.0-87.2)	3.2 (2.4-4.7)
	7½ yrs.	Unblanched	5.0 (4.5-5.8)	100.6 (99.9-103.6)	84.1 (83.9-84.2)
Corn					
Golden Cross	2½ yrs.	Unblanched	45.6 (43.7-46.5)	105.1 (104.6-105.8)	46.3 (44.9-48.1)
	2½ yrs.	Blanched	8.4 (7.8-9.2)	110.2 (109.0-111.6)	1.6 (1.2-1.95)
	1952 0 (Fresh) Crop	Unblanched	0	106.1 (104.7-106.9)	3.4 (3.2-3.6)
Lima Beans					
Fordhook	6 yrs.	Unblanched	3.7 (3.6-4.0)	116.7 (114.8-118.3)	11.4 (11.4-11.4)
	6 yrs.	Blanched	0	115.1 (113.3-116.7)	3.0 (3.0-3.0)
	1952 0 (Fresh) Crop	Unblanched	0	112.3 (111.5-113.4)	3.0 (2.6-3.4)
Snap Beans					
Tendergreen	6 yrs.	Unblanched	24.1 (23.8-24.3)	101.2 (101.0-101.5)	91.2 (90.5-91.9)
	6 yrs.	Blanched	0	92.5 (90.5-94.4)	2.7 (2.4-3.0)
	1952 0 (Fresh) Crop	Unblanched	0	98.7 (98.6-98.8)	8.4 (7.6-9.2)
Spinach					
Unknown variety	6 yrs.	Unblanched	33.6 (32.4-35.6)	114.6 (114.0-117.9)	43.4 (41.0-46.1)
	6 yrs.	Blanched	0	114.8 (113.0-115.7)	0
Peas					
Topper	7½ yrs.	Unblanched	39.9 (38.5-41.8)	97.7 (97.2-98.6)	107.9 (106.4-109.3)
Alderman	7½ yrs.	Blanched	0	106.8 (105.8-108.7)	4.4 (3.7-5.1)
Alderman	7½ yrs.	Unblanched	32.6 (32.4-32.7)	95.4-103.9	103.0-104.3

[1]Samples were run in 3 to 5 replicates. Table shows averages and extreme values, the latter in parentheses.

It is known that both lima beans *(3)* and asparagus *(4)* are low in lipoxidase. This could account for the low and negative peroxide values reported for the crude lipids obtained from these unblanched vegetables. It is well to note that the work by Reiser and Fraps *(3)* was presumably done on the dry seeds, which may possibly alter the result somewhat. The fact that the crude lipid from unblanched lima beans is also low in acid is interesting.

Corn is the only vegetable which shows a peroxide number in the crude lipid extracted from the stored material which was blanched before having been placed in frozen storage. If this finding is confirmed, it seems to suggest that in this vegetable non-enzymic peroxidation can take place. This particular point is being studied further, together with the progressive development of peroxides and acids in several vegetables. The results will be reported in a later paper.

It may be that the peroxide values are partially qualitative, in the sense that variation in technique may yield higher values.

In a previous publication *(2)* the ether extracted pea powder from unblanched stored peas was reported to have given no rancid flavor. It was concluded that all the off-flavor was in the lipid fraction, and, therefore, ether extractable. At that time, 48 hours was used as the period of extraction. In the present studies, the extraction period was cut down to 24 hours, as discussed before, and in most cases, some off-flavor remained in the residue from the unblanched material. It will be necessary to determine whether longer extraction is necessary to remove all the compounds contributing to off-flavor, or whether some vegetables produce compounds, insoluble in ether, which have this property.

The lipid contents of the several vegetables are shown in Table 3.

TABLE 3

Crude lipid content of vegetables

Asparagus	0.15 to 0.30%
Corn	0.75 to 0.84%
Lima Beans	0.33 to 0.49%
Peas	0.34 to 0.48%
Snap Beans	0.09 to 0.14%
Spinach	0.27 to 0.58%

SUMMARY

A continuation of the work reported in 1951 on peas included a study of the crude lipids extracted after frozen storage from asparagus, corn, lima beans, snap beans, and spinach, and some additional work on the crude lipid extracted from peas. A table showing the crude lipid contents of the vegetables studied is provided.

It was demonstrated that the peroxides found in the crude lipid extracted from raw vegetables after periods of frozen storage tend to decrease in quantity on standing.

It was concluded that iodine number is not of much value as an index of changes in the crude lipid fraction.

Corn, snap beans, and spinach were found to be similar to peas in that high peroxide numbers and high or fairly high acid numbers were found when the crude lipids extracted from the unblanched frozen stored vegetables were analyzed. Similar crude lipid prepared from asparagus gave a high acid number, but no peroxide value, whereas the crude lipid extracted from raw lima beans gave low peroxide and acid numbers.

The crude lipid from corn which was blanched before having been placed in frozen storage showed a low peroxide number after 2½ years' storage. This suggests that non-enzymic peroxidation is perhaps taking place.

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FURTHER STUDIES ON THE EFFECT OF EXPERIMENTALLY INOCULATING CANNED BREAD WITH SPORES OF *CLOSTRIDIUM BOTULINUM*[a, b]

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From the data obtained in previous experiments designed to test the safety of canned bread *(3)*, military specifications for pH and moisture were written for this product; namely, that the pH of the crumb shall not exceed 4.8 and the total moisture content of the finished bread shall not exceed 33%. These specifications were assumed to be readily obtainable by commercial bakers in producing canned bread for the Armed Forces. The desired pH level was attained by adding the proper quantity of edible lactic acid to the sponge water. Bakers in some instances, however, encountered difficulty in staying below the allowable maximum moisture level. In the present study experiments were designed to test the safety of canned bread from the botulism hazard at several moisture levels above the maximum stated in the Specifications. Spores of the same six strains of *Clostridium botulinum* (three each of types A and B) tested previously were used as the inoculum. In addition several uninoculated control cans were included with each experimental series. One half of the canned bread was adjusted with edible lactic acid to have a final pH near 4.8. The other half was adjusted with sodium hydroxide solution to yield a final pH near 5.8. The moisture levels aimed at in the baked product were 34, 36, 38, and 40%. The actual levels, as determined by analysis, were somewhat different and moisture variations were encountered among samples baked from similar doughs.

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[a] The experiments reported in this investigation were carried out in cooperation with the staff of the Quartermaster Food and Container Institute for the Armed Forces.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 482 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of Defense.

MATERIALS AND METHODS

Approximately 700 No. 2½ size cans (401 × 411) of bread were prepared, baked, canned, and stored at the Quartermaster Food and Container Institute in Chicago by members of the Institute staff. The bread was prepared using the following formula:

Ingredients	% based on weight of flour
Flour[1]	100.00
Water[2]	60.00-72.00
Yeast, compressed	2.00
Salt	1.75
Sugar	4.00
Shortening[3]	4.00
Milk powder[4]	2.00
Edible lactic acid (80%) or .125 N sodium hydroxide solution[5]	

[1] Flour—low protein, short patent, hard winter wheat.
[2] Water—the amount of water added was varied to obtain four different moisture levels in the baked product.
[3] Shortening—hydrogenated shortening, 100 hr. stability.
[4] Milk powder—high acid buttermilk solids.
[5] Edible lactic acid, 80% concentration, manufactured by E. I. du Pont de Nemours. Edible lactic acid was used to adjust one half of the samples to pH 4.8 and sodium hydroxide solution was used to adjust the other half of the samples to pH 5.8.

A sufficient volume of a phosphate buffer spore suspension containing 60 million spores of equal numbers of Types A and B, *Cl. botulinum* (3 strains of each) was inoculated into each of the sponge waters to give an inoculum of 1,000,000 spores per can (10 oz. of dough, yielding approximately 9½ oz. of bread).

The following strains of *Cl. botulinum* were used in the inoculum:

Strain	Type	Obtained from
457	A	Dr. L. S. McClung, Indiana University
62A	A	Dr. K. F. Meyer, Hooper Foundation, University of California
109	A	Dr. K. F. Meyer, Hooper Foundation, University of California
169	B	Dr. K. F. Meyer, Hooper Foundation, University of California
12033	B	National Canners Association
126.7	B	Department of Bacteriology, The University of Chicago

The uninoculated bread samples (lots 1-4 and 9-12) were prepared first to avoid contamination with spores of *Cl. botulinum*. The bread was baked at 450°F. for 30 minutes. Immediately upon removal from the oven the cans were sealed, stacked one deep in racks, and then air cooled. The sealing operation was made on pilot plant sealing equipment.

All cans were tested for vacuum immediately before storage by means of a "flip tester." Before opening a can for analysis an absolute vacuum reading was taken with a vacuum gauge by puncturing the lid.

All samples were stored at 90°F. and were subjected to weekly cycling at 40°F. for 16 hours, following which they were returned to the 90°F. incubator. This cycling was done to simulate practical storage conditions favoring condensation of moisture in portions of the can.

At the end of each storage period one can was selected at random from each group to provide a sample for pH analysis. The pH determinations were made on 15 g. of interior crumb suspended in 100 ml. of freshly boiled and cooled distilled water. A Beckman potentiometer, Model H2, was used for these analyses.

Three loaves from each uninoculated and 10 loaves from each inoculated group were analyzed for appearance, consistency, odor, and toxin as soon as practicable after baking. Similar numbers of cans from each group were examined after 1 and 2 weeks, and after 1, 3, 6, and 12 months of storage at 90°F. (only bread from experimental lots initially adjusted to near pH 5.8 were tested after 1 and 2 weeks of storage). One can from each uninoculated and 2 cans from each inoculated group were analyzed for the presence of viable spores at each of these intervals. All bread cans and samples were stored in the refrigerator until needed.

After the top of each can had been cleaned with alcohol and flamed, the end with the headspace was opened (cans from which samples for spore recovery analyses were to be obtained were opened under a hood using aseptic technique). Each loaf was observed for appearance, consistency, and odor. The bread was mixed with a sterile wooden tongue depressor during the addition of 550 ml. of sterile 0.85% saline (making a 1:3 dilution of the bread). The can was then covered with a sterile glass plate. The following day 20-ml. portions of the fluid from the settled bread suspensions were transferred to sterile centrifuge tubes. After centrifuging these samples at high speed, the clear supernatant fluids were used for mouse inoculations. Test mice were injected intraperitoneally with 1 ml. of this fluid, and were observed for 4 days. Where a test mouse died, a series of 4 mice was inoculated with the toxic sample. All mice received 1 ml. of the sample, and one of the 3 control mice received in addition either 0.1 ml. of Type A, 0.1 ml. of Type B, or 0.3 ml. of ABC antitoxin.

Ten ml. samples of the bread suspension from 1 can of each control group (1 can of 3) and from 2 cans of each inoculated group (2 cans of 10) were inoculated to flasks containing 90 ml. of beef heart medium for determination of the presence of viable *Cl. botulinum* spores. Decimal dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) of the bread suspensions were also made in tubes containing 9 ml. of beef heart medium to determine the approximate number of viable spores. The inoculated beef heart flasks and tubes were heated at 80°C. for 10 minutes to destroy any viable vegetative cells and were then incubated at 86°F. for 10 days. Following incubation the clear centrifuged supernatant fluid from the cultures was tested for toxicity in mice to determine whether any viable spores had survived baking and storage. Each mouse received, by intraperitoneal injection, 0.5 ml. of the broth. Where a test mouse died a series of 4 test mice was injected as described above, using a 1:10 saline dilution of the toxic sample as the test fluid.

Moisture analyses on representative samples from the contents of 3 cans of each experimental group were conducted according to the combination air and vacuum drying method specified in *Cereal Laboratory Methods (1)*.

RESULTS

Vacuum readings. All cans opened during the initial analysis (no storage at 90°F.) had a vacuum of 30 inches. In general, it was found that with an increase in storage time, there was an increase in the number of cans having reduced vacuum, both in the inoculated and uninoculated groups. It was also observed that cans which contained toxin usually had a decreased vacuum. Some of the inoculated samples with high moisture content showed positive pressure in the cans after 6 months of storage (lots 15 and 16). At the end of 12 months of storage, the vacuum readings on both uninoculated and inoculated samples initially adjusted to near pH 4.8 were still very satisfactory in most cans, although a few had no vacuum or a slight pressure. Among similar samples adjusted to near pH 5.8 less than one-fourth of the cans had vacuum readings in excess of 20 inches.

Appearance, consistency and odor. At the initial analysis the cut surface of the bread appeared almost white. Prior to the 3-month analysis, the contents of all cans had a normal appearance and consistency. At the end of 3 months of storage at 90°F., all uninoculated samples were satisfactory, but the contents of 3 cans from among the inoculated groups had changed to a grayish-brown color. At 6 months the bread in 8 cans was grayish-brown, and after 12 months of storage the contents of an additional 3 cans had changed to a definite grayish-brown color. This discoloration occurred in samples from both uninoculated and inoculated groups. By the end of 12 months of storage at 90°F. the interiors of all other canned loaves were a light brown.

In general, it was found that with an increase in storage time, there was an increase in the number of samples having a sour odor. The contents of a few cans from the higher moisture groups were sour after only 1 week of storage. After 3 months of storage some of the samples had a sweet odor as contrasted to the sour odor noted previously in many of the higher moisture samples. With increasing storage time, the predominant odor changed from sour to pungent sweet. At the 6-month analysis, practically all inoculated samples in the groups initially adjusted to near pH 5.8 had off odors, and many samples in the other lots, both inoculated and uninoculated, had fermentation odors. After 12 months of storage the odors coming from the freshly opened

cans were very similar to those observed at 6 months. Stale odors were noted in all cans that did not have the sweetish aroma.

Among the inoculated samples analyzed at 3 months, two of 3 samples with a light grayish-brown color had soggy areas along the can seams. The contents of these cans had a very pungent sweetish odor. At the 12-month analysis one inoculated can contained a similar soggy area.

pH. The pH readings on the contents of representative cans, taken at the initial analysis (no storage at 90°F.) and on similar samples taken after 12 months of storage at 90°F., are shown in Table 1.

TABLE 1

pH of canned bread stored at 90°F.

Lot No.	Spore inoculum	Storage time	
		None	12 months
1	None	4.9	4.7
2	None	4.9	4.6
3	None	4.9	4.9
4	None	4.6	4.5
5	None	5.8	5.4
6	None	5.8	5.6
7	None	5.8	5.5
8	None	5.8	5.3
9	1,000,000 per can	5.1	4.9
10	1,000,000 per can	5.0	4.9
11	1,000,000 per can	4.9	4.9
12	1,000,000 per can	5.1	4.9
13	1,000,000 per can	5.7	5.4
14	1,000,000 per can	5.7	5.4
15	1,000,000 per can	5.8	5.3
16	1,000,000 per can	5.8	5.5

Examination of the above tabulated data showed that the pH readings on the samples initially adjusted to near pH 4.8 were only slightly changed after 12 months of storage. The readings on the samples adjusted to near pH 5.8 had dropped from 0.1 to 0.5 below their corresponding initial values in the series inoculated with the spores of *Cl. botulinum* as well as in the uninoculated controls.

Moisture. Moisture determinations were made on 3 cans of each of the 16 lots of bread. A mean moisture level for each lot was determined from these analyses and the individual values are also listed to give the probable range of variation among the samples in each lot. (See Table 2.)

Botulinum toxin. Samples from the uninoculated lots (1-8) and from the inoculated lots (9-12) which were initially adjusted to approximately pH 4.8 were negative for botulinum toxin at all analyses.

The number of samples (of 10 analyzed), from each inoculated lot initially adjusted to approximately pH 5.8, which contained botulinum toxin are shown in Table 3.

Viable spore count. All beef heart flasks inoculated with 10 ml. of a 1 to 3 saline dilution from the control bread samples were negative for botulinum toxin, which indicated that the uninoculated samples were free from contamination with *Cl. botulinum* spores.

Analyses from the presence of viable *Cl. botulinum* spores were conducted on the contents of 2 cans from each group of 10 inoculated samples. With the exception of one can from lot 10 at the 12-month analysis, all cans examined had at least 3 to 30 viable spores per g. of bread. In a number of cans viable spores were found in larger numbers. The positive samples at the higher dilutions are shown in Table 4.

These data showed that in some instances the probable number of viable spores present was greater than those surviving the baking process. A trend toward greater numbers was also shown by the fact that the number of cans which yielded positive

TABLE 2

Moisture analyses on representative cans of canned bread used in this study

Lot No.	Percent moisture, individual samples			Percent moisture average	Percent moisture range
	a	b	c		
1	35.68	35.68	34.00	35.12	34.00–35.68
2	35.62	35.82	34.78	35.41	34.78–35.82
3	36.83	37.41	36.32	36.85	36.32–37.41
4	39.48	39.61	38.18	39.09	38.18–39.61
5	34.83	35.65	33.80	34.76	33.80–35.65
6	36.37	36.98	35.80	36.38	35.80–36.98
7	37.15	38.12	36.70	37.32	36.70–38.12
8	39.94	41.07	41.50	40.84	39.94–41.50
9	36.20	35.65	36.22	36.02	35.65–36.22
10	37.06	38.06	38.16	37.76	37.06–38.16
11	37.15	38.63	39.21	38.33	37.15–39.21
12	40.79	40.92	39.94	40.55	39.94–40.92
13	35.81	35.80	35.57	35.73	35.57–35.81
14	38.15	37.32	37.76	37.74	37.32–38.15
15	38.13	39.51	39.49	39.04	38.13–39.51
16	39.48	41.28	41.22	40.66	39.48–41.28

samples at the higher dilutions became steadily greater at each subsequent analysis, except that fewer positives were noted after 12 months of storage. This increase was most marked among samples from lots having the higher moisture levels.

At the 3- and 12-month analyses it was observed that in several of the higher dilutions in the beef heart tubes only type B toxin was detected, which indicated that type A spores had reached a very low level in cans that had a probable total viable spore count in excess of 30 per g. of bread.

After 12 months of storage at 90°F., analyses for the presence of *Cl. botulinum* spores showed that all inoculated cans examined (except 1 can of lot 10, which had less than one viable spore in 3 g.) contained at least 3 to 30 viable spores per g. of bread. In several cans larger numbers were found. These data showed that the probable number of viable spores remaining after 12 months of storage at 90°F. was low in all samples in which growth and toxin production had not occurred. In all groups of samples initially adjusted to approximately pH 4.8 the most probable number remaining was in the range of 3 to 30 per g. In the lowest moisture group at pH 5.8 similar spore levels remained. The higher numbers present in the other 3 groups initially at pH 5.8 were probably spores arising from the multiplication of vegetative cells during active growth and toxin production. The number of spores added to all inoculated samples was approximately 3,500 per g.

There were no toxic samples among those initially adjusted to approximately pH 4.8. (See Table 5.) The numbers of samples in the group inoculated with *Cl. botulinum*

TABLE 3

Clostridium botulinum toxin in canned bread experimentally inoculated with Cl botulinum spores and after storage at 90°F.
(Initial pH of bread—approximately 5.8)

Lot No.	Moisture range	No. of cans containing toxin after storage for: (10 cans inoculated with 1,000,000 spores per can for each time interval)						
		None	1 wk.	2 wks.	1 mo.	3 mos.	6 mos.	12 mos.
	%							
13	35.57–35.81	0	0	0	0	0	1	0
14	37.32–38.15	0	0	0	4	6	8	10
15	38.13–39.51	0	0	6	9	10	10	10
16	39.48–41.28	0	0	8	9	10	10	10

TABLE 4

Presence of viable spores of Clostridium botulinum, Types A and B, in canned bread after baking at 450°F. for thirty minutes (1:3 dilution of canned bread)

Storage time at 90°F.	Lot No.	No. of cans in which viable spores were present in 0.1 ml. samples[1]	No. of cans in which viable spores were present in 0.01 ml. samples[1]	No. of cans in which viable spores were present in 0.001 ml. samples[1]
None	9	0	0	0
	10	0	0	0
	11	2[3]	0	0
	12	1	1[4]	0
	13	1	0	0
	14	0	0	0
	15	0	0	0
	16	0	0	0
1 week[2]	13	1	1	0
	14	1	0	0
	15	2	0	0
	16	0	0	0
2 weeks[2]	13	1	1	0
	14	1	1	0
	15	0	1	0
	16	2	0	0
1 month	9	1	0	0
	10	0	0	0
	11	1	1	0
	12	1	0	0
	13	0	1	0
	14	0	0	0
	15	2	0	0
	16	2	2	2[5]
3 months	9	1	0	0
	10	0	1	0
	11	0	0	0
	12	0	0	0
	13	0	0	0
	14	1[6]	1[6]	1[6]
	15	1	0	0
	16	1[7]	2[6]	2[6]
6 months	9	1	0	0
	10	0	0	0
	11	0	0	0
	12	0	0	0
	13	1	0	0
	14	2	1	0
	15	1	0	0
	16	2	1	0
12 months	9	0	0	0
	10[8]	0	0	0
	11	0	0	0
	12	0	0	0
	13	0	0	0
	14	1	1	0
	15	1	2	1
	16	2[7]	2[6]	1[6]

[1] Cans were tested in duplicate.
[2] Samples adjusted to near pH 4.8 were not analyzed after one and two weeks of storage.
[3] Presence in 0.1 ml. samples means from 30 to 300 viable spores per gram of bread.
[4] Presence in 0.01 ml. samples means from 300 to 3,000 viable spores per gram.
[5] Presence in 0.001 ml. samples means from 3,000 to 30,000 viable spores per gram.
[6] Type B toxin only.
[7] One sample had Type B toxin only.
[8] One can contained between 3 and 30 spores per gram; the second can had less than one spore in 3 grams.

spores (lots 9-12) which had decreased vacuum readings after storage was no greater than among similar uninoculated cans (lots 1-4). Changes in pH among these samples were less than 0.2, except for 2 uninoculated samples of lot 2, each of which had pH decreases of 0.2. One of these cans had a vacuum reading of 4 inches and the other a pressure reading of 1 lb.

Among samples initially adjusted to approximately pH 5.8, there were some toxic samples in groups incubated at 90°F. for periods of 2 weeks or longer. The toxic samples are indicated in Table 5. The number of samples in the inoculated group (lots 13-16) which had decreased vacuum after storage was only slightly higher than among similar uninoculated cans (lots 5-8). The number of cans with lowered vacuum readings was more than twice as great among the series at pH 5.8 than among the group initially adjusted to approximately pH 4.8. Similarly, changes in pH were greater among samples of the group initially adjusted to approximately pH 5.8 than among the other samples. Decreases in pH of as much as 0.6 were observed and some rises of a smaller magnitude were also noted. In general, fluctuations in pH were just as prevalent among the uninoculated samples as among the inoculated ones, and no correlation of drop in vacuum with a shift in pH level was established. A drop in vacuum was observed in all toxic cans. In some cans this drop was only a few inches, but lower vacuum readings were recorded for the toxic samples stored for longer periods. The shift in pH was not always in the same direction in the toxic samples; therefore, this criterion cannot be used to predict toxicity. Furthermore, other changes occurred in the contents of the stored cans which caused losses in vacuum that could not be related to the development of toxin.

DISCUSSION

The results of this study showed that canned bread initially adjusted to approximately pH 4.8 is not a satisfactory medium for growth and toxin production of *Cl. botulinum* even at moisture levels near 40%. Data on bread of a similar composition adjusted to approximately pH 5.8 revealed that a lowered pH prevented growth of *Cl. botulinum*. After storage at 90°F. for 1 year, all samples having moisture levels below 36% were nega-

TABLE 5

Changes in pH and vacuum in relation to toxin production of Clostridium botulinum in representative samples of canned bread stored for various intervals at 90°F.

Lot No.	Moisture average, percent	Initial		Storage Intervals at 90°F.											
				1 week[1]		2 weeks		1 month		3 months		6 months		12 months	
		pH	Vac.	pH	Vac.	pH	Vac.	pH	Vac.	pH	Vac.	pH	Vac.	pH	Vac.
1	35.12	4.9	30					5.0	30	4.9	30	4.9	30	4.7	30
2	35.41	4.9	30					4.9	30	4.7	4	4.8	30	4.6	+1
3	36.85	4.9	30					4.9	30	4.7	30	4.6	10	4.9	30
4	39.09	4.6	30					4.6	30	4.5	30	4.5	22	4.5	27
5	34.76	5.8	30	5.8	30	5.5	30	5.2	30	5.6	1	5.4	30	5.4	30
6	36.38	5.8	30	5.8	30	5.6	30	5.9	24	5.8	4	5.8	17	5.6	17
7	37.32	5.8	30	5.8	30	5.8	28	5.9	7	5.8	26	5.8	17	5.5	18
8	40.84	5.8	30	6.0	30	6.0	20	6.0	13	5.8	16	5.5	15	5.3	16
9	36.02	5.1	30					5.0	30	5.0	1	5.1	30	4.9	30
10	37.76	5.0	30					5.0	29	5.1	30	5.0	30	4.9	25
11	38.33	4.9	30					4.9	30	5.0	30	5.0	30	4.9	30
12	40.55	5.1	30					5.0	30	5.1	27	5.0	30	4.9	30
13	35.73	5.7	30	5.9	30	5.6	30	5.7	29	5.9	12	5.8	25	5.4	25
14	37.74	5.7	30	5.6	30	6.0	15	5.6	27	5.6	30	5.8	15[2]	5.4	0[2]
15	39.04	5.8	30	6.0	1	6.0	14	5.5	25[2]	6.0	19[2]	5.6	12[2]	5.3	4[2]
16	40.66	5.8	30	5.7	30	6.2	27[2]	6.0	3	5.9	14[2]	5.6	+3[2]	5.5	7[2]

[1]Samples initially adjusted to approximately pH 4.8 were not tested after 1 and 2 weeks of storage at 90°F.

[2]Samples that contained botulinum toxin.

tive for toxin production. One toxic sample in the lowest moisture group at the 6-month analysis could possibly have had a moisture level slightly above the moisture range determined by analyzing 3 representative cans from each group of samples, because these analyses clearly showed that there were varying amounts of moisture lost from the cans during the baking process. If there were no chances for the pH level in the canned bread to be shifted by the growth of other microorganisms during storage, then, canned bread of any practical moisture content would be safe from the botulism hazard. However, puncturing and subsequent contamination of cans of bread during rough handling is not a remote possibility under adverse conditions. During additional storage at warm temperatures, growth of these contaminants could shift the pH level to a range favorable for initiation of growth of *Cl. botulinum* spores. Therefore, a moisture level of 36%, which proved to be inhibitory at pH 5.8, must be the highest level permissible under any circumstance. Furthermore, the data on pH, physical appearance, vacuum, and odor showed that moisture levels above 36% would not be feasible from the standpoint of other undesirable changes in the canned product during prolonged storage even if the possibility of a botulism hazard did not exist. There was no visible breakdown of the normal bread structure of samples in which toxin was produced; therefore, toxic loaves cannot be detected by observing their appearance or consistency.

A marked drop in vacuum at the 2-week and 1-month analyses was usually correlated with the development of a sour odor. Most of the cans showing decreased vacuum readings were in the groups which had the two higher moisture levels tested. This indicated a greater degree of activity in the samples with more moisture. After longer periods of storage some of the samples showing a marked decrease in vacuum developed a sweetish odor. In many instances this odor was noted in cans which had botulinum toxin present, but this aroma was not exclusively characteristic of toxic samples.

The viable spore counts showed that some spores had survived the baking process in all bread samples tested. In general, they survived at a level of 3 to 30 spores per g., but in some instances higher numbers were recovered. Some samples that contained toxin had more spores present at the later analyses than did similar samples that had no storage at 90°F. This indicated that part of the greater number of viable spores had arisen from the multiplication of vegetative cells which had subsequently formed spores. In the bread samples at approximately pH 4.8 the number of samples which yielded viable spores at the higher dilutions tested became progressively fewer as the storage period was prolonged. Halvorson *(2)* suggested that some of the spores may have germinated under conditions which were not sufficiently favorable to permit the growth of the vegetative cells.

SUMMARY

Sixteen lots of bread were prepared, baked, and vacuum packed in cans. All samples were stored at 90°F. and were cycled weekly for 16 hours at 40°F. for intervals up to 12 months. One-half of the samples were adjusted to approximately pH 4.8 and the remainder to near pH 5.8.

Moisture levels of approximately 34, 36, 38, and 40% were studied at each of the two different pH levels.

The results of the toxin analyses showed that with bread initially adjusted to pH levels near 4.8 no toxin was produced even at moisture levels near 40%. However, in similar bread initially adjusted to approximately pH 5.8 toxin was produced in some cans after 2 weeks of storage, and a higher percentage of positive samples was encountered at each subsequent analysis. After 12 months of storage all samples with moisture levels above 36% which were adjusted to approximately pH 5.8 contained toxin.

The pH readings on the samples initially adjusted to near 4.8 showed only slight changes after 12 months' storage. The readings on the samples initially adjusted to approximately pH 5.8 had dropped from 0.1 to 0.5 below their corresponding initial values in the series inoculated with the spores of *Cl. botulinum* as well as in the uninoculated controls.

At the end of 12 months of storage, the vacuum readings on both uninoculated and inoculated samples initially adjusted to near pH 4.8 were still very satisfactory in most cans, although a few had no vacuum or a slight pressure. Among similar samples adjusted to approximately pH 5.8 less than one-fourth of the cans had vacuum readings in excess of 20 inches.

After 12 months of storage at 90°F., analyses for the presence of *Cl. botulinum* spores showed that all inoculated cans examined (except 1 can of lot 10, which had less than one viable spore in 3 g.) contained at least 3 to 30 viable spores per g. of bread. In several cans larger numbers were found. Some viable spores remained in all inoculated cans examined; hence, safety is assured only if the bread continues to be inhibitory to the growth and toxin production of these spores (i.e. no other changes occur in the can which will shift the pH level to a range favorable for growth).

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PECTIC CONSTITUENTS IN RIPE AND UNRIPE FRUIT*

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Fruit pectins consist primarily of polyanhydrogalacturonic acid units in unbranched chains with varying amounts of nongalacturonide carbohydrate materials such as araban and galactan probably in or closely associated with them. Usually 80% of the carboxyl groups are esterified with methyl alcohol, and the remaining 20% are free acids or salts. In some pectins a few of the secondary alcohol groups are esterified as acetyl esters. In addition to these variables in composition, pectins vary in their molecular sizes. These variations in composition and average molecular size probably affect texture of fruits, consisteney and gel strength of processed fruits, and rate of drying and subsequent rehydration of fruits.

A study of the pectic substances in ripe and unripe fruits has been made, including characterizations of certain of the chemical and physical properties of pectins extracted from specific fruit tissues. Pectic enzymes are generally assumed to be the cause of changes in texture during ripening of fruits. Since the enzyme pectinesterase occurs almost universally in fruits *(6)*, its activity was not determined. Polygalacturonase, not generally found in fruits *(6, 7)*, was assayed to determine, if possible, whether its presence could be demonstrated in peaches, pears, and avocados.

EXPERIMENTAL

Selection of samples. The fruits examined in the present study were obtained from a fruit grower. Thus they were orchard-run samples, picked from the same trees at the same times. About 60 pounds of Elberta peaches were selected for color and firmness. One lot of green-hard fruits was selected to represent unripe peaches. A lot of yellow-soft fruits was selected as the ripe sample. No pressure tests were made. Twenty-five pounds of Bartlett pears (all green and hard) were selected for uniform color and size, free from bruises or abrasions. The stems had been detached at the absciss layer during picking and it was assumed that the fruits were of the same maturity. These unripe fruits were immediately subjected to analysis. The remaining fruits were stored at 25°C.(77°F.) and after 8 days they were uniformly colored yellow and had the softened texture of fully ripened pears. These were regarded as the ripe pears and were then subjected to analysis. One box of size 30 unripe-green-hard Fuerte avocados was divided into two portions. The unripe portion was analyzed immediately. The remaining fruits were stored at 25°C.(77°F.) for 6 days. The fruits were then soft to the touch and the skin could be separated easily from the flesh. All of the fruits were superficially comparable in their degree of ripeness. These were considered to be ripe avocados and were then processed as described.

Extraction procedures. Ripe and unripe fruits of the same variety were extracted with boiling alcohol; the dried sugar-free material, referred to herein as "marc," was then analyzed directly and the pectic substances extracted from the marc by mild means. Pectic substances, assumed to be representative of those present in the fruit,

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were then isolated and characterized by their anhydrouronic acid, methoxyl, and acetyl contents, and by viscosity (a measure of molecular size related to jellying power).

Methods. Analytical methods employed were for the most part those previously published by Owens *et al. (11)*. Anhydrouronic acid (AUA) was determined on fresh and dry sugar-free material by a colorimetric method using carbazole *(8, 9)*.

After removal of adsorbed alcohol *(5)* methoxyl analyses were run on dry fruit residue and pectin by Clark's modification *(1)* of the Zeisel method.

Acetyl determinations were made by a modification *(13)* of Clark's method for acetyl *(2)*.

Viscosities of the pectic substances were determined with an Ostwald-Cannon-Fenske viscosimeter by the technique described by Owens *et al. (11)*.

Polygalacturonase assays were conducted by the method of Jansen and MacDonnell *(4)*.

Preparation of fruit marc. Sound fresh Elberta peaches, ripe and unripe, were halved and the pits removed. Two kilograms of each were sliced into 6 liters of boiling 95% ethanol and heated for 30 minutes with agitation. The alcohol was drained from the marc and evaporated to dryness for later tests for sugars and uronic acids. The marc was washed once with 2 liters of 95% ethanol and then dried in air. It was then ground to pass 40 mesh and extracted in a Soxhlet with 80% ethanol in the extraction flask. The extracted residue was further extracted with acetone and the marc was then dried in air.

Dry sugar-free marc was prepared from ripe and unripe Bartlett pears and Fuerte avocados (minus the seeds) in the manner described here. Analyses were made directly on the alcohol-acetone-extracted marc from these fruits.

RESULTS AND DISCUSSION

Characterization of fruits studied. Elberta peach pectin extracted from ripe and unripe fruits has a degree of esterification of above 86% and an acetyl content of about 3%. (See Table 1.) The intrinsic viscosity of the pectin extracted from ripe fruit was about half that of unripe fruit. No polygalacturonase enzyme activity was found. Bartlett pears and Fuerte avocados undergo very great changes in texture during ripening. The pectin from unripe fruits from these species had intrinsic viscosities showing high molecular weights and both showed a high degree of esterification. Pear pectin was found to have an acetyl content of about 3.5%, which is higher than pectins from most fruits previously examined. The acetyl content of avocado pectin was less than 2%. Upon ripening, the intrinsic viscosity of the pectin from pears and avocados dropped to a low value, the degree of esterification dropped to about 40%, and the acetyl content remained unchanged.

The anhydrouronic acid contents of ripe peaches, pears, and avocados were essentially the same as those of unripe fruits. The anhydrouronic acid content of the peach marc from both ripe and unripe fruits was 29% and the avocado marc from ripe and unripe fruits analyzed alike at 11%. Unripe and ripe pear marc analyzed 22 and 17%, respectively. A considerable quantity of pectin from ripe pears dissolved in the 63% ethanol used to extract sugars and inactivate enzymes. This fact was demonstrated by evaporation of the alcoholic solutions, treating with pectinase and testing for the presence of galacturonic acid by paper chromatography *(10)*. After enzymic hydrolysis the pear fraction contained sufficient galacturonic acid to account for the difference in anhydrouronic acid between the ripe and the unripe pear marc. The 63% alcoholic extracts of the other fruits treated in a like manner showed only traces of galacturonic acid.

Analyses for methoxyl and acetyl esters directly on the mare samples failed to reveal significant differences between ripe and unripe materials.

Pectic substances. Pectic substances are macromolecular; therefore, plant tissue from which they are to be extracted must be maintained in a swollen condition to permit them to diffuse out. The presence of carboxyl groups in pectin offers opportunities for cross linking with other macromolecules through polyvalent cations. Acid or cation acceptors such as sodium ethylene diamine tetra-acetic acid (Versene) sodium polymetaphosphate or sodium oxalate, are required. Hydrogen bridges between associated high polymers must be dissociated by heating or by use of a suitable hydrogen-bond-breaking agent. These three general requirements for pectin extraction were partially satisfied with water as the extraction medium, Versene as the calcium-sequestering agent, vigorous agitation to disintegrate the material, and allowance of several hours for the macromolecules to diffuse out of the insoluble cell-wall matrix.

Extraction of pectin. One part of dry sugar-free mare was suspended in 20 parts of 0.5% Versene[b] solution at pH 6.0 and stirred vigorously for 4 hours at 25°C.(77°F.). The suspension was then filtered with suction on a bed of diatomaceous silica filter aid. Since the yield of pectin contained in the filtrate and the original weight of slurry were known, a correction for the pectin remaining in the filter cake in equilibrium with the solution was made to obtain the total pectin extracted. The pectin-containing filtrate was acidified to pH 2 with hydrochloric acid and poured into 2 volumes of 95% alcohol. The precipitated pectin was separated from the solution and after washing out the excess acid, the whole was suspended in acetone. Acetone served to further dehydrate and remove traces of ethanol that interfers with methoxyl determinations. Drying in air at 25°C.(77°F.) overnight removed the acetone and the pectins were then ground to pass 40 mesh and analyzed. Results of these analyses on the moisture-free basis are summarized in Table 1.

In the case of unripe fruits about one-third of the total anhydrouronic acid was extracted by the 0.5% Versene and recovered as pectin. The two-thirds of pectin substance remaining in the fruit mare under these conditions was assumed to have a high molecular weight. Otherwise it had chemical characteristics similar to those of the extracted material *(11)*. The chief differences between these fractions have been found to lie in their molecular weights. Diffusion of such macromolecules is very slow at 25°C., and incomplete extraction results. In ripe fruits the molecular weight of the pectin is much lower, the rate of diffusion of molecules is much more rapid, and the efficiency of the extraction procedure is increased. In the case of the ripe peaches and pears, 50 to 60% of the total AUA present was extracted but only 60% of the material was recovered as an alcohol precipitate. With the ripe avocado pectin extract, no precipitation of pectin occurred until the alcohol content was increased to 70%, but no additional material was obtained when the ethanol content was increased from 70 to 80%.

[b]Obtained from the Bersworth Chemical Co., Framingham, Mass. Mention of products by specific manufacturers does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

TABLE 1

Yield and analysis of Versene-extracted pectin from marc of ripe and unripe fruits

Material	Anhydrouronic acid		Analyses on pure pectin basis		Intrinsic viscosity
	Extracted %	Precipitated in 63% ethanol %	Degree of esterification %	Acetyl content[1] %	
Unripe peach	33	94	86	3.0	10.0
Ripe peach	51	61	89	2.9	4.3
Unripe pear	37	92	89	3.3	6.0
Ripe pear	60	58	43	3.7	1.5
Unripe avocado	37	68	100	1.1	3.9
Ripe avocado	60	41[2]	32	2.0	1.0

[1]Acetyl content as CH_3–CO.
[2]Precipitated in ethanol concentration of 70%

Fruit pectins can usually be purified to contain 70 to 80% anhydrouronic acid and up to 12% methoxyl. The nonuronide materials are not assumed to contribute significantly, except as a diluent, to the chemical properties or to the viscosity. Some of the analytical values in Table 1 are expressed on a basis of 100% anhydrouronic acid on the extracted material for reasons of clarity, and the methoxyl contents are expressed as degree of esterification.

Esterification. Peach pectin from both unripe and ripe fruits has a degree of esterification of 86%, and an unusually high acetyl content of 2.9%. These characteristics did not change during ripening. The most drastic change observed upon ripening in the case of peaches is the reduction of the molecular size of the extraction pectin as indicated by loss in intrinsic viscosity from more than 10 to 4.3.

The pectin of unripe pears is somewhat similar to that of unripe peaches. In ripe pears, however, pectin shows a loss in degree of esterification from 89 to 43% and a drop in intrinsic viscosity from 6.0 to 1.5. A slight increase in acetyl accompanies ripening.

Avocados ripen very rapidly (within 7 days) and have an extremely soft texture when ripe. They were found to have pectin somewhat similar to that of the other unripe fruits. In ripe fruit, the degree of esterification of the pectin had dropped from 100 to 32% and the intrinsic viscosity from 3.9 to 1.0.

Until recently, the importance of acetyl groups in pectins, as factors in gelation, was unknown. However, it has been shown that acetyl in quantities smaller than that encountered here (2.3%) can affect gelation of pectins or even prevent gelation completely *(3, 12)*.

Changes in degree of esterification affect both the solubility and the gelation properties of pectin. The degrees of esterification of pectins from unripe peaches and pears examined here seem to be similar to those of most fruit pectins, while that of unripe avocado was unusually high. As pears and avocados ripened, the degrees of esterification of their pectins dropped to 43 and 32%, respectively. These are low methoxyl pectins and as such are sensitive to precipitation by calcium and other cations. This loss in degree of esterification seems to be unrelated to the change in

texture of these fruits, because the loss of molecular weight of the pectins can more than account for the observed changes. However, the loss of ester groups indicates that the enzyme, pectinesterase, has mixed with the pectic substances and caused deesterification.

The pectins from these fruits, after ripening, had intrinsic viscosities of 4.3, 1.5, and 1.0, respectively. These correspond to approximate jelly grades of 220, 60, and 0. While the intrinsic viscosity of the pectin of ripe peaches remained high, it had decreased by more than 50% compared with pectin from unripe fruits. The molecular sizes of the pectins of ripe pears and avocados were so low in the experiments reported here that if the pectins are responsible for the texture of fruits they probably would not be able to maintain a firm texture or contribute very much to the consistency of processed foods.

Determination of the polygalacturonase activity of ripe and unripe fruit. Pectin-glycoside-splitting enzymes such as polygalacturonase have not been widely reported to occur in fruits, with the exception of tomatoes. Kertesz *(7)* found no activity in unripe tomatoes and reported that it is not always detectable in ripe tomatoes. Subsequently polygalacturonase activity has been found in many varieties of ripe tomatoes by various workers. The fruits examined here were subjected to a polygalacturonase assay in an attempt to account for the pectin degradation that occurred during ripening. One-hundred-gram samples of each fruit were blended at high speed for 2 minutes in an electrical blender, each with 100 g. of a 10% sodium chloride solution. Ten-gram samples of the slurry were used as sources of enzyme, each in 50 ml. of 1% polygalacturonic acid solution at pH 5.0, and at intervals 5-ml. aliquots of digest were removed and analyzed for reducing power *(4)*.

No polygalacturonase activity, as measured by increase in reducing power, was found in the unripe fruits or in ripe peaches. Weurman *(14, 15)* reported that an inhibitor occurs in some varieties of pears which may prevent the action of polygalacturonase. In the case of ripe Bartlett pears and Fuerte avocados, considerable polygalacturonase activity was observed. About 0.001 and 0.035 millimole of polygalacturonide-glycosidic linkages were split per hour per gram of fruit. This unusually high polygalacturonase activity seems to be directly related to the extensive lowering of the molecular sizes of pectin in ripe pears and avocados.

SUMMARY

Pectic substances were extracted by mild means from ripe and unripe Elberta peaches, Bartlett pears, and Fuerte avocados, precipitated with ethanol, and characterized by analysis.

Peach and pear pectin from both ripe and unripe fruits had unusually high acetyl contents of 3.0 and 3.5%, respectively. The degree of esterification of the pectin with methyl ester groups was above 86% in the unripe fruits. The degree of esterification dropped to below 40% after the pears and avocados ripened and the molecular sizes of all of the pectins decreased.

No pectin-degrading polygalacturonase activity could be demonstrated in the unripe fruits, but ripe pears and avocados showed activities of 0.001 and 0.035 millimole of bonds split per hour per gram of fruit, respectively.

These results suggest that, in the fruits studied, pectic enzymes come in contact with the pectic substances during ripening and hydrolyze them to materials of lowered molecular sizes. The degraded materials are then less effective in maintaining firm structures in fruits and less important in contributing to the consistency of processed foods.

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INHIBITION OF THE GROWTH OF *CLOSTRIDIUM BOTULINUM* BY ACIDIFICATION

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Some low acid canned foods suffer a serious loss in quality when heat processed sufficiently to destroy spores of *Clostridium botulinum*. Among them are certain vegetables commonly packed in brine, such as artichoke hearts, brussels sprouts, onions, and cauliflower; low acid foods mixed with an acid food, such as egg plant in tomato sauce, zucchini in tomato sauce, and pineapple-rice pudding; juices, such as vegetable juice cocktail; and sauces, such as spaghetti and mushroom sauces. With products such as these, canners have resorted to pH control as a means of inhibiting the growth of *Cl. botulinum* and reducing the thermal process necessary to prevent spoilage. Control may be achieved by the addition of an edible acid, such as citric or acetic, or by the incorporation of an acid ingredient, such as a tomato product, a fruit juice, or crushed or pureed fruit.

Dozier *(1)*, working with vegetative cells of 37 strains of *Cl. botulinum* in phosphate buffered double strength veal infusion-2% Difco peptone, found a growth range of pH 4.87 to pH 8.89, while with spores of 19 strains in the same medium she found a growth range of pH 5.01 to pH 8.89. Other than this, little work has been reported on the pH levels necessary to inhibit the growth of *Cl. botulinum*.

The question may be raised that in most of the experiments described in this paper citric acid was used to adjust the pH, and that the pH limits chosen might not be valid if other edible acids were used. The two acids commonly added to canned foods for pH control purposes are citric and acetic. Reid *(4)*, Levine and Fellers *(2)*, Nunheimer and Fabian *(3)*, Shillinglaw and Levine *(5)*, and others, working with a variety of bacteria and yeasts, have demonstrated that acetic acid is more growth inhibitory and germicidal than citric acid at comparable pH levels. They have also shown that all the edible organic acids are more toxic to bacteria and yeasts than are the highly dissociated inorganic acids, such as HCl, at the same pH. It seems safe to assume that the same relationship would apply to the germination of bacterial spores. Consequently the highest pH at which germination of *Cl. botulinum* spores was found to be inhibited when a food was acidified with citric acid should also inhibit germination when acetic acid is used.

As a result of unpublished data obtained by the National Canners Association Western Branch Laboratory in 1927-1931 from studies on the

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acidification of artichokes to prevent the growth of *Cl. botulinum*, the maximum pH level for control purposes was agreed to be 4.6, although for commercial operating conditions canners were advised to keep the pH below 4.5. This level has been used by the industry for many products for more than 20 years, and no single instance has been recorded of failure to inhibit the growth of food poisoning bacteria.

There has been some evidence that the pH level for inhibition of the growth of *Cl. botulinum* is not the same for all foods, and some products acidified to below pH 4.5 are not accepted readily by consumers. As a result, this laboratory undertook a study of the pH levels at which spores of *Cl. botulinm* would germinate, grow, and produce toxin in a variety of food products. This paper may be considered a progress report in which data obtained over a period of several years are presented.

EXPERIMENTAL PROCEDURE

Products. Where the product was not homogeneous it was macerated and mixed in a Waring blender—with the addition, if necessary, of a small amount of water. It was then divided into small lots to which various amounts of acid (citric or acetic) or NaOH were added to give different pH levels. The pH adjusted material was tubed in 22 mm. × 175 mm. pyrex test tubes and autoclaved for 20 minutes at 250°F.(121°C.). After autoclaving, the pH of one tube from each lot was determined. Duplicate tubes were then inoculated with 20-25 million spores of *Cl. botulinum*, types A and B, suspended in 0.25 ml. sterile tap water, and previously detoxified by heating for 5 minutes in boiling water. The spores were thoroughly mixed with the medium, which was then covered with sterile vaseline. The inoculated tubes together with uninoculated controls were incubated for an indefinite period (at least a year) at 30°C. and observed for evidence of growth.

When growth as evidenced by gas formation occurred, the tube was held in the incubator for at least one more week before a toxicity test was made. Toxicity tests were made on the product having the lowest pH in which growth occurred, and also after a long incubation period on the product having the highest pH in which no growth was apparent. In the later experiments the pH of the uninoculated control was determined at the time growth was observed in inoculated tubes, because it was found that the pH tended to change somewhat during incubation, particularly at the higher pH levels.

To determine whether any sample was toxic it was filtered by centrifugation through a Seitz filter pad, and 0.2 ml. of the filtrate was inoculated intraperitoneally into a mouse. If symptoms of botulism were observed, a second mouse was inoculated in the same manner with 0.2 ml. of filtrate which had been heated for 5 minutes in boiling water. If the second mouse showed no symptoms of botulism, symptoms observed in the first mouse were considered to have been produced by heat labile *botulinum* toxin. In some instances the tests had to be repeated several times because of non-specific reactions. The results are shown in Tables 1 and 2.

The cultures of *Cl. botulinum* used were from the following sources: 62A and 213B, and composite A and composite B, all from the collection of Dr Karl F. Meyer, G. W. Hooper Foundation for Medical Research. Composite A included Nos. 3, 33, 62, 73, 78, 172, and 240, and composite B included Nos. 4, 32, 34, 113, 115, 169, and 213. These were selected from a larger number of isolates because they were found to produce considerable toxin in culture media. The other two cultures were isolated by the National Canners Association Western Branch Laboratory, No. 12885-3A from asparagus and No. 12033-4B from shrimp.

Culture media. In order to avoid testing large numbers of foods to determine the lowest possible pH at which spores of *Cl. botulinum* might germinate, it was decided to experiment with culture media, on the assumption that a suitable culture medium would be more likely to provide the most favorable conditions for spore germination.

TABLE 1

Growth of Clostridium botulinum in products adjusted to various pH levels

(pH adjusted with citric acid or NaOH except where otherwise indicated)

Legend: + = gas or toxin production (in appropriate columns)
− = no gas or no toxin production (in appropriate columns)
WT = weak toxin (mouse showed symptoms of botulism but recovered)

Product	Adjusted pH	pH at time of growth	Visible growth (gas formation)		Toxin production		Incubation Days at 30°C. for growth	
			62A	213B	62A	213B	62A	213B
Green chili peppers (fresh) (1)	4.55		−	−				
	4.85		−	−				
Original pH 4.55	5.03	4.73	−	−	−			
(pH usually 4.8-5.5)	5.27	5.01	+	+	+	+WT	20	11
	5.50	5.3	+	+		+	11	11
	6.47	5.96	+	+			8	8
Green chili peppers (fresh) (2)	4.57	4.40	− −	− −				
	4.86	4.62	− −	− −				
Original pH 4.57	5.04	4.77	− −	− −	−			
(pH usually 4.8-5.5)	5.32	4.93	+ +		+		20	
	5.53	5.20	+ +	+ +		+	11	11
	5.70			+ +		+		11
	6.40	5.70	+	+ +			8	8
Pimientos (fresh) (1) (red peppers)	4.63		−	−				
	4.97		−	−	−			
Original pH 4.63	5.27	5.07	+	+	+	−	63	29
(pH usually 4.5-5.3)	5.48	5.16	+	+	+	−	11	25
	6.0	5.62	+	+		+	6	6
Pimientos (fresh) (2) (red peppers)	4.62	4.50	− −	− −				
	4.97	4.70	− −	− −				
Original pH 4.62	5.25	5.03	+ +	+ −	+	−	63	29
(pH usually 4.5-5.3)	5.45	5.07	+ +	+ −	+	−	11	25
	6.00	5.51	+ +	+ +		+	6	6
Eggplant with tomato sauce (canned) (Mixture of 2 parts product to 1 part water was used) Original pH 4.53	4.56		−	−				
	4.61		−	−				
	4.75		−	−				
	4.92		−	−				
	5.13		−	−	−	−		
Pineapple-rice pudding (canned) Original pH 4.41	4.59		−	−				
	4.80		+	−	+		51	
	4.94		+	+		+	16	23
	5.16		+	+			9	14
	5.30		+	+			7	10
	5.47		+	+			6	6
Pork and beans (1) (pH of sauce adjusted to various levels before mixing with beans and sterilizing)	4.12		−	−				
	4.35		−	−				
	4.73		−	−				
	4.93		+	+	+	+	15	25
	5.02		+	+			11	13
	5.20		+	+			6	7
	5.31		+	+			4	4
	5.67		+	+			4	4
Pork and beans (2) (pH of sauce adjusted to various levels before mixing with beans and sterilizing)	4.35		−	−				
	4.57		−	−				
	4.73		−	−	−			
	4.97	5.00	+	+	+	+	16	19
	5.20	5.22	+	+		+		
	5.36	5.33	+	+				
	5.53	5.53	+	+				

TABLE 1 (Concluded)

Product	Adjusted pH	pH at time of growth	Visible growth (gas formation)		Toxin production		Incubation: Days at 30°C. for growth	
			62A	213B	62A	213B	62A	213B
Pork and beans (3) (pH of sauce adjusted to various levels before mixing with beans and sterilizing)	4.34		−	−				
	4.59		−	−				
	4.72		−	−	−			
	4.95	5.00	+	+	+	+	9	12
	5.19	5.22	+	+		+	5	7
	5.33	5.33	+	+			5	5
	5.53	5.53	+	+			5	5
Spaghetti sauce (canned) Original pH 4.11	4.57		−	−				
	4.78		−	−				
	4.96		−	−	+	−		
	5.23	5.20	+	+	+	+	17	28
	5.40	5.37	+	+			10	18
	5.59	5.58	+	+			7	11
Spaghetti, with tomato sauce and cheese (canned) Original pH 4.47	4.61		−	−				
	4.81		−	−				
	5.00	4.96	+	+	+	−	14	33
	5.20	5.24	+	+		+†	7	11
	5.40	5.40	+	+	(control also died in 2 tests)		6	7
	5.60	5.59	+	+				
Spaghetti, with meat (canned) Original pH 4.55	4.53		−	−	−	−		
	4.70		−	−				
	4.91		+	+	+	−	15	12
	5.32		+	+	+	+	4	4
Prune pudding (canned) Original pH 4.68	4.61		−	−				
	4.74		−	−				
	4.85		−	−				
	4.98		−	−				
	5.23		−	−				
	5.44		−	+	−	−		66
Strained pears with farina (canned) Original pH 3.97	4.61		−	−				
	4.78		−	−				
	5.07		−	−				
	5.17		−	−				
	5.42		−	+		−		66
	5.53		−	+		−		66
Strained plums with farina (canned) Original pH 3.57	4.76		−	−				
	5.00		−	−				
	5.20		−	+	−	−		156
	5.48		+	+	+	−	33	29
	5.62		+	+			75	26
	5.71		+	+			40	40
Vegetable juice cocktail (canned) (1) Original pH 4.37	4.38		−	−				
	4.56		−	−				
	4.67		−	−				
	4.83		−	−				
	5.03		+	+	+	+	31	17
	5.32		+	+			11	11
Vegetable juice cocktail (canned) (2) Original pH 4.17	4.47		−	−				
	4.68		−	−				
	4.84		−	−	+			
	5.04		+	−	+	−	16	
	5.20	5.27	+	+		+		15
	5.42	5.39	+	+			5	12
Zucchini with tomato sauce (canned) Original pH 4.80	4.50		−	−				
	4.62		−	−	−	−		
	4.76		−	−				
	4.82		−	−				
	4.86		+	+	+	+	18	33
	5.18		+	+	+	+	5	5

TABLE 2

Growth of Clostridium botulinum in banana puree adjusted to various pH levels

Legend: + = gas production or toxin present (in appropriate columns)
− = no gas production or no toxin present (in appropriate columns)

Product	Adjusted pH	pH at time of growth	Visible growth (gas formation) Cp. A[1]	Cp. B[2]	Toxin production Cp. A	Cp. B	Incubation Days at 30° C. for growth Cp. A	Cp. B
Banana puree (fresh) (acidified with acetic acid)	4.19		−	−				
	4.28		−	−				
	4.41		−	−				
	4.49		−	−				
	4.61		−	−				
Banana puree (fresh) (acidified with citric acid)	4.13		−	−				
	4.27		−	−				
	4.39		−	−				
	4.50		−	−				
	4.62		−	−				
	4.75		−	−				
	7.00		+	+	+	+	60	60
Banana puree (NaOH added) Original pH 4.72-4.82 (aseptically canned product)	5.02		−					
	5.20		−					
	5.61		−					
	5.72		−					
	6.02		+		? Results		90	
	6.20		+		? Confusing because of death of controls		90	

[1] Composite A = Mixture of 7 strains of *Cl. botulinum*, type A.
[2] Composite B = Mixture of 7 strains of *Cl. botulinum*, type B.

Three media were selected for study: corn steep liquor, Wheaton's beef heart-casein broth, and Yesair's pork infusion broth. The following are the formulae for these media:

Corn Steep Liquor

To tap water adjusted to pH 11, add 0.3% technical casein, sifting the casein slowly into the water and grinding to prevent the formation of lumps.

Add 1% corn steep liquor and adjust the pH to 7.2.

Autoclave for 20 minutes at 121°C.(250°F.).

The preparation of the corn steep liquor should be as follows:

Add 55 parts corn steep liquor (raw) to 4 parts water.
Adjust pH to 8.5, using either 40% NaOH or slaked caustic.
Heat to 65°F. for one-half hour.
Add one handful of Johns Manville "Celite" per 6 liters.
Filter while hot on a Buchner funnel into a suction flask.

For the medium use the filtrate, which should contain 40 to 52% total solids. Keep in refrigerator.

Wheaton's Beef Heart-Casein Broth

Prepare beef heart infusion from 450 g. ground fat free meat per liter distilled water, simmering for one hour.

Take 1,000 ml. beef heart infusion and adjust to pH 8.9.

Add 5 g. isoelectric casein. Then add the following:

Tryptone (Difco) . . .	10.0 g.	K_2HPO_4 (anhydrous) . .	4.0 g.
Gelatin	10.0 g.	Sodium citrate	3.0 g.
Glucose	0.5 g.		

Tube or flask with meat particles added.

Autoclave for 20 minutes at 121°C.(250°F.).

Yesair's Pork Infusion Broth

Prepare pork infusion, 450 g. ground fat-free pork per liter distilled water, simmering for one hour. Strain through cheesecloth. Add the following:

Peptone (Difco) . . .	5.0 g.	Glucose	1.0 g.
Tryptone (Difco) . . .	1.5 g.	Soluble starch	1.0 g.
K_2HPO_4 (anhydrous) .	1.25 g.		

Cool, and adjust pH to 7.4-7.6.

Autoclave for 20 minutes at 121°C.(250°F.).

The 3 media were treated in the same way as the food products as far as pH adjustment, inoculation, incubation, and testing of cultures were concerned. Results are shown in Table 3.

TABLE 3

Growth of Clostridium botulinum in culture media adjusted to various pH levels

Legend: + = gas production or toxin present (in appropriate columns)
− = no gas production or no toxin present (in appropriate columns)

Medium	Adjusted pH	pH at time of growth	Strain and Results					
			12885 −3A	62A	78A	12033 −4B	115B	213B
Corn steep liquor (pH adjusted with citric acid)	4.42		−	−	−	−	−	−
	4.53		−	−	−	−	−	−
	4.57		−	−	−	−	−	−
	4.70		−	−	−	−	−	−
	4.80	4.77	−	−	−	−	−	−
	4.90	4.98	+	−	−	−	−	−
	5.00	5.05	+	−	−	−	+	−
	5.10	5.12	+	+	+	+	+	−
	5.20	5.28	+	+	+	+	+	+
			Composite			Composite		
			7 type A strains			7 type B strains		
			Growth (gas)		Toxin	Growth (gas)		Toxin
Wheaton's medium (pH adjusted with citric acid)	4.40		−			−		
	4.55		−			−		
	4.60		−			−		
	4.65		−			−		
	4.75		−			−		
	4.78		−			−		
	5.02		+		−	−		
	5.08	5.09	+		+	−		
	5.20	5.22	+		+	+		
	5.30		+			+		
Yesair's pork infusion broth (pH adjusted with HCl)	4.39		−			−		
	4.47		−			−		
	4.54		−			−		
	4.66		−			−		
	4.70		−			−		
	4.80		−			−		
	4.90		−			−		
	5.00	4.98	+		+	+		+
	5.10	5.06	+		+	+		+

DISCUSSION

In general, the type A strains of *Cl. botulinum* are either equally tolerant or more tolerant to acid than the type B strains, at least as far as growth with toxin production is concerned. In a number of instances growth of type A strain with toxin production occurred at a lower pH than was the case with the type B strain. However, in one group of products, strained fruit puddings with farina, growth of the type B strain occurred at a much lower pH than with the type A strain, although no toxin was produced.

In several products toxin was present at the next pH level below the lowest at which gas was formed, showing that gas production is not a necessary indication of growth and toxin formation in a food product.

The results show that the pH level of 4.6, used as the maximum for control purposes in products where acidification is relied on to inhibit the growth of *Cl. botulinum*, has provided an ample safety factor. The lowest pH at which growth occurred in the experiments reported here was 4.80 (pineapple rice pudding). However, it may be that growth without gas production may be possible at a lower pH level in this and some other products. For all products tested, pH 4.7 appears to be about the lowest level at which germination and growth of *Cl. botulinum* spores can be expected. Some products, however, seem to be less favorable for the germination of spores of *Cl. botulinum* than others.

The results in culture media (Table 3) were unexpected, in that growth was inhibited at a higher level of pH than in certain food products. In corn steep liquor growth occurred at pH 4.98, but not at 4.77; in Wheaton's beef heart-casein medium at pH 5.02 but not at 4.78; and in Yesair's pork medium at pH 4.98 but not at 4.90. This confirms the work of Dozier *(1)* who obtained no germination of *Cl. botulinum* spores in a veal infusion-peptone medium below pH 5.01. It should be noted also that there is considerable variation among strains in two of the three media tested.

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A HISTOLOGICAL AND HISTOCHEMICAL STUDY OF BEEF DEHYDRATION. IV. CHARACTERISTICS OF MUSCLE TISSUES DEHYDRATED BY FREEZE-DRYING TECHNIQUES[a]

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Previous studies *(14, 15)* showed that muscle tissues dehydrated at 65° or 70°C. rehydrated poorly with little indication of structural reconstitution. Shrinkage of the muscle fibers and the tissues as a whole, resulting in complete obliteration of interfibral spaces, occurred during dehydration of beef muscle. In addition, alterations in the physical and/or chemical nature of the muscle tissue probably also occurred at these temperatures. These were some of the reasons for the observed limited rehydratability of muscle tissues dehydrated conventionally by warm-air methods.

It soon became apparent that a dehydration technique was needed that would minimize both physical and chemical changes and distortion of the structural and spatial organization of the tissue. The technique which best fulfills these requirements is that of freeze-drying. This technique was first introduced by Altmann *(1)* as an ideal means of tissue fixation for histological work, and was further elaborated and improved by Gersh *(8)*. It has since been widely used in the preparation of medical and pharmaceutical products as well as in the food industry *(6, 7, 9)*. In essence, this technique removes moisture from the tissue in a frozen state by lyophilization at such low temperatures that little chemical or physical changes can occur. Hence, no denaturation of any of the constituents of a cell or tissue was observed with this technique (See. IV, 9).

The experiments reported here were conducted with two objectives:

(a) Freeze-drying as a technique for dehydrating muscle tissues was evaluated against previous results from the application of conventional warm-air methods. The structural pattern of the frozen-dried tissue as related to its rehydratability was emphasized. Further, the influence of pre-freezing temperature on the histology of the lyophilized tissue and its subsequent rehydration and reconstitution was ascertained.

(b) It was found that partially lyophilized samples contained a core of essentially unchanged raw tissue enclosed in a shell of almost completely dried tissue. These samples were used for studying the mechanism of rehydration and reconstitution of dehydrated muscle tissue. Additional studies indicated also that the moisture in such material could be driven off at

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elevated temperatures in an oven without seriously reducing the rehydratability of the dried product.

MATERIAL AND METHODS

Strips of muscle tissue ¼-inch thick and weighing about 5 g. were taken from light weight commercial carcasses approximately 4 days after slaughter. Both the *Biceps femoris* and the *Semimembranosus* muscles of the round were utilized, the majority of the samples from the former.

Samples were pre-frozen at one of three temperatures by the following method: placed into individual containers in dry ice (−80°C.) for ½ hour; immersed for 3 minutes in isopentane cooled down to −150°C. by liquid nitrogen; or placed into a cold room (−17°C.) for 6 hours.

Immediately after the period of pre-freezing, the samples were placed into a laboratory lyophilizer and frozen-dried for varying lengths of time. No more than 10 samples were run at one time in order to stay within the capacity of this apparatus. In some preliminary experiments, thermometers were inserted into samples to record temperature rise against time of dehydration. Generally, the temperature never reached the point of thawing until moisture removal was nearly complete. Vacuum during freeze-drying ranged from 0.001 to 0.3 mm. mercury.

Ground samples of raw meat were dried in a vacuum oven for 18 hours at 65°C. to determine moisture content. Dehydrated tissue was rehydrated by immersion in demineralized water for 15-30 minutes at 23°C.

Controls (raw) were taken from the same muscles as the experimental group and fixed in Zenker-formol. No fixation of the dehydrated samples was needed as they were put into absolute ethanol. Rehydrated samples were fixed in 10% neutral formalin. All samples were processed for celloidin embedding. Sections were cut at 10 μ and stained with hematoxylin and Van Gieson's for microscopic study and muscle fiber diameter determination. Sections of the same samples were also studied for distribution of potassium by the Carere-Comes technique *(3)*.

The core-containing partially lyophilized samples were processed as follows: when taken out of the lyophilizer the entire sample was weighed to determine its residual moisture content. It was then divided into two halves, each containing part of the core. One of them was fixed in Zenker-formol for histological processing; the other half was weighed and put in a vacuum oven for a period varying from 3-6 hours. Three oven temperatures, 23°, 45°, and 70°C., were used. At the end of this oven dehydration, the sample was weighed and again divided into two portions, each of which included a part of the core. One of these two portions was put into absolute ethanol for histological processing; the other was rehydrated and its weight was taken both before and after rehydration. The rehydrated tissue was then fixed in 10% neutral formalin for histological processing. From these, complete data were obtained on moisture and structural changes in a sample throughout the process. For each of the stages, sections (both longitudinal and transverse) for histological and histochemical determinations were made available. All sections for histological studies were stained with hematoxylin and Van Gieson's. Muscle fiber diameter measurements were made on both the core and periphery. Potassium movement was determined by the Carere-Comes technique *(3)*.

RESULTS AND DISCUSSION

A. **Effect of pre-freezing temperature on the completely frozen-dried muscle tissue.** All frozen-dried muscle tissues, regardless of pre-freezing temperature, had a pinkish-tan color. The color probably was due to a modification of myoglobin which normally colors meat bright red. The most significant thing, however, was that the tissue underwent no change in shape, form, or volume when moisture removal was accomplished by the freeze-drying technique.

Microscopically, frozen-dried muscle tissues had a number of characteristics in common. First, a system of spaces invariably developed which thoroughly interpolated the tissue. Most muscle fibers shrank without

losing individuality or cross-striations. Detailed structures are described below.

1. Pre-frozen at −150°C.

Lyophilized muscle tissue pre-frozen at this temperature was most nearly like normal in structural organization. There was no distortion in spatial relationship or alteration in the normal proportion of muscle fibers and the interfibral spaces, both endomysial and perimysial (Figure 3A). Although the muscle fibers did not shrink, there were tiny spaces inside the muscle fibers (Figures 3A and 3H). These intracellular spaces were the only observable evidence that freezing had taken place. These spaces have long been recognized as artifacts *(9)*; this was substantiated since frequently they were retained even after the tissue was rehydrated (Figure 3C).

The size of the intracellular spaces (ice loci) was smallest at the edge of the sample and became progressively larger toward the center (from left to right, Figure 3H). These gradients were caused by temperature differences in the tissue along a peripheral-central path at the time of pre-freezing. The muscle fibers were tightly packed at the tissue edge, as evidenced by complete obliteration of endomysial spaces (extreme left of Figure 3H). (The cracks marked "X" in the figure are probably artifacts due to processing.) Ice spaces were absent from these muscle fibers indicating that freezing at this region was so rapid that the resulting ice crystals were probably microscopic in size and amorphous (see *8*).

2. Pre-frozen at −80°C.

Pre-freezing at this temperature altered the proportion of muscle fibers and interfibral spaces although total tissue volume remained unchanged. This change was the result of a considerable shrinkage by the muscle fibers. The fibers were shriveled irregularly along their course so that they were no longer of a uniform width (Figure 3D). Thick (150 μ) sections of this tissue showed that the continuity of the individual muscle fibers was not broken. Any appearance of transverse gaps or longitudinal splitting seen from standard 10-μ sections is due entirely to cutting through a fiber along a curvature or band. Also, the irregular pattern of this shriveling suggests that a mixed intra- and intercellular freezing had probably determined the morphological pattern of the tissues pre-frozen at this temperature. This shriveling and absence of spaces inside these shriveled muscle fibers combined, indicate that freezing at this temperature started out to be intracellular but became more and more intercellular. Thus, the freezing pattern, though predominantly of the latter type, was clearly intermediate between those obtained at −150°C. and −17°C.

It should be emphasized that while muscle fiber shrinkage had contributed to the increase of spaces, the enlarged spaces nevertheless retained the same characteristics of the original endomysial and perimysial spaces.

3. Pre-frozen at −17°C.

Lyophilized tissue pre-frozen at this temperature presented yet another distinct pattern. It was characterized by uniform reduction in the diameter of muscle fibers and the emergence of an entirely new space system, which no longer bore any resemblance to endomysial spaces either in size or location. Furthermore, these uniformly shrunken (not shriveled) muscle fibers were grouped together into small bundles, and fibers in the bundles were packed without a trace of endomysial space (Figure 3F). This morphology appeared to have resulted from slow but complete intercellular freezing. Conceivably, while the endomysial spaces were either retained at −150°C. or greatly enlarged at −80°C., at −17°C. a displacement of these same spaces occurred. During this displacement, most endomysial spaces disappeared (note closely packed muscle fibers in Figure 3F), while only a few of them had increased tremendously in size. The latter were interfibral spaces marking the positions occupied by ice loci.

Interpreted on the basis of established principles *(5, 6, 7, 8, 10)*, the above results would mean that the structural pattern of frozen-dried muscle tissue is a function of rate of freezing which, in turn, is determined by the pre-freezing temperature used. As freezing rate decreases with rising temperature, freezing changed from an intracellular to an intercellular pattern with decreasing frequency of loci but increasing size of ice crystals.

Histological observations in connection with frozen meat and the influence of freezing temperature on palatability, storage, drip loss, etc., have been made *(3, 5, 10, 12, 13)*. However, none of this work involved dehydration of the frozen products.

REHYDRATION AND RECONSTITUTION

Rehydration of frozen-dried muscle tissue differed completely from that of similar tissues dried by warm air at 65° or 70°C. previously reported *(14, 15)*. Upon immersion in demineralized water at room temperature, the tissue almost immediately changed color from pinkish-tan to reddish-brown as it became covered with tiny bubbles. Both these phenomena seemed to be simultaneous with water penetration. In most cases, tissues remained afloat after 15 minutes but occasionally a rehydrated sample would sink to the bottom of the beaker. There was every indication that rehydration was rapid for frozen-dried muscle tissue; most of the water was absorbed in the first 3 minutes after immersion. Rehydration did not affect the total volume of the tissue. Rehydrated tissue closely resembled untreated raw meat in gross appearance.

Rehydratability was measured by two criteria: increase in both moisture content (Figure 1) and muscle fiber diameter upon rehydration. The data (Table 1) show an over-all high degree of rehydratability for all lyophilized beef muscles to a moisture level of 84-89% of raw, and a muscle fiber diameter of over 89-98% of raw. Muscle rehydratability as high as this had not been hitherto obtained in our laboratories. It is notable that this high rehydratability was characteristic of frozen-dried muscle tissue in general.

Two essential factors probably were responsible for this high rehydratability of frozen-dried muscle tissue, i.e., lack of protein denaturation, and presence of a permeating system of spaces. The former was attributed to the fact that freeze-drying was carried out at temperatures so low that biochemical activities were reduced to a minimum, if not completely. As a result, there was no apparent denaturation of protein (actomysin) as evidenced by (a) freedom from case-hardening; (b) absence of muscle fiber merging; and (c) maintenance of distinct cross-striations. This is important from a rehydration standpoint because chemical changes may prevent rehydration, even though adequate amounts of water are in contact with the muscle fibers. This is shown by the fact that frozen-dried steaks stored for several months at 100°F. do not rehydrate well even though the space system has not been altered *(16)*.

The space system, on the other hand, provided necessary avenues for conveying water to the muscle fibers, thus playing an equally indispensable role in rehydration. These spaces, characteristic of all frozen-dried muscle tissues, owe their origin first to an initial "fixation" of a given piece of meat by pre-freezing. For instance, the data (Tables 1 and 2) show that while moisture content decrease was progressive up to 10 hours of lyophilization, no corresponding continual decrease in muscle fiber diameter was observed (maximum reduction was reached within three hours of lyophilization). Also, this might explain why no correlation between moisture content and muscle fiber diameter of the degree reported previously *(14, 15)* was obtained from the present samples.

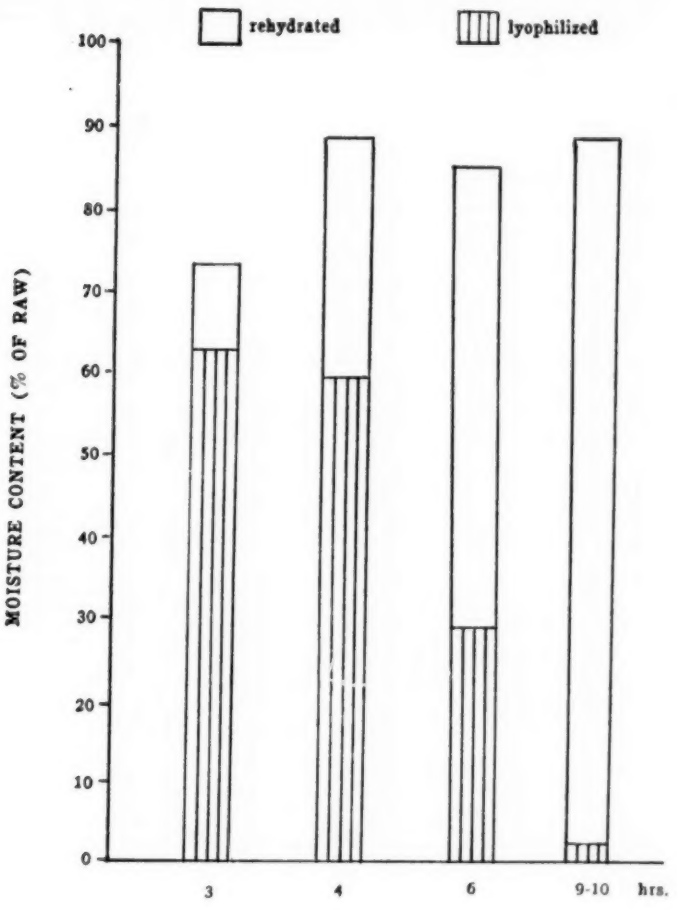


Figure 1. The average moisture content of lyophilized and rehydrated beef.

Later, during lyophilization, moisture loss from the tissue without altering the sample volume readily accounted for the emergence of spaces. Conceivably, they arose to take the place previously occupied by ice crystals, wherever they occurred.

Furthermore, there are numerous indications, as follows, of a direct quantitative relationship between the space in frozen-dried tissue and its capacity to reabsorb water and reconstitute:

(a) Samples pre-frozen at −17°C. had the largest amount of interfibral space and also the greatest degree of muscle fiber diameter recovery upon rehydration. They also had the fastest initial water penetration during rehydration.

TABLE 1

Averages and standard deviation of moisture content and fiber diameter values of lyophilized and rehydrated beef samples pre-frozen at different temperatures

No. of samples	Muscle	Lyophilization time (hrs.)	Pre-freezing temperature °C.	Average moisture content (%)			Average muscle fiber diameter (μ)		
				Raw	After lyophilization	After rehydration	Raw	After lyophilization	After rehydration
1	*Biceps femoris*	10	−150	76.3	3.5	63.9	34.2	39.7	33.5
6	*Biceps femoris*	10	−150	74.5	2.5 ± 4.3	66.4 ± 3.8	39.7	36.4 ± 4.0	36.9 ± 2.1
4	*Biceps femoris*	9	− 80	72.6	2.0 ± 4.4	63.7 ± 1.8	40.2	23.2 ± 1.4	35.8 ± 2.9
10	*Biceps femoris*	9	− 80	73.4	0.0	64.5 ± 4.5	37.8	25.0 ± 2.0	35.9 ± 2.8
6	*Semimembranosus*	24	− 17	73.3	0.25 ± 1.0	62.2 ± 10.7	34.8	21.8 ± 1.3	33.9 ± 1.5

TABLE 2

Averages and standard deviation of moisture content and muscle fiber diameter of samples, incompletely lyophilized and followed in some cases by oven dehydration at three different temperatures

No. of samples	Lyophilization time (hr.)	Pre-freezing temperature °C.	Oven drying		Average moisture (%)				Average muscle fiber diameter (μ)						
			Temp. °C.	Time (hr.)	Raw	After lyophilization	After oven dehydration	After rehydration	Raw	After lyophilization		After oven drying		After rehydration	
										Core	Peri.	Core	Peri.	Core	Peri.
3	3	− 80	....	0	72.6	18.2 ± 9.5	....	61.9 ± 5.0	40.2	....	22.5 ± 1.9	....	....	....	35.0 ± 0.5
3	6	− 80	....	0	72.6	2.0 ± 4.4	....	63.7 ± 1.8	40.2	....	23.2 ± 1.4	....	....	....	35.8 ± 2.9
3	3	− 80	23	22	72.6	43.3 ± 15.2	1.7 ± 2.6	55.1 ± 12.5	40.2	37.2 ± 4.2	23.5 ± 0.7	28.5 ± 2.9	23.4 ± 1.6	29.4 ± 6.7	38.3 ± 2.9
3	6	− 80	23	19	72.6	18.2 ± 9.3	4.9 ± 4.2	61.9 ± 0.2	40.2	31.9	25.8 ± 7.0	22.0	22.2 ± 2.7	"	34.9 ± 1.9
10	3	− 80	45	3	73.9	22.6 ± 10.0	3.4 ± 2.5	61.3 ± 5.9	35.2	35.4 ± 1.9	23.4 ± 2.2	25.5 ± 2.6	22.7 ± 2.2	30.5 ± 3.1	33.9 ± 2.5
1	3	−150	45	6	76.3	54.6	2.5	50.0	34.2	23.9	35.5	21.5	32.5	26.0	30.9
1	6	−150	45	3	76.3	30.1	11.5	64.6	34.2	32.4	36.2	26.9	38.0	26.1	31.6
1	3	−150	....	0	76.3	54.6	....	50.0	34.2	....	35.5	....	....	....	30.9
3	4	−150	....	0	74.5	41.6 ± 11.1	....	65.7 ± 3.9	39.7	....	42.2 ± 5.0	....	....	....	36.4 ± 1.8
1	6	−150	....	0	76.3	30.1	....	64.6	34.2	....	39.7	....	....	....	33.5
2	3	− 80	45	6	76.3	28.1 ± 35.0	6.7 ± 2.7	64.7 ± 3.7	34.2	35.7 ± 2.2	25.2 ± 0	26.5 ± 2.6	26.0 ± 1.4	31.1 ± 0.1	30.9 ± 3.1
7	6	− 80	45	3	75.7	20.8 ± 9.8	7.2 ± 1.8	66.8 ± 4.4	37.0	....	25.0 ± 1.3	27.0	24.3 ± 1.8	29.8 ± 2.3	35.3 ± 2.8
5	6	− 80	45	3	75.5	27.1 ± 10.6	7.3 ± 2.7	64.6 ± 3.4	38.2	....	....	32.7 ± 10.3	25.1 ± 2.3	29.2 ± 3.3	37.6 ± 4.0
10	4.5	− 80	70	3	75.4	15.7 ± 14.9	1.8 ± 1.3	69.3 ± 2.9	35.8	....	....	22.6 ± 2.0	23.5 ± 2.4	32.0 ± 0.2	30.0 ± 0.2

(b) Recovery of endomysial spaces increased with increasing pre-freezing temperature, whereas interfibral space retention in rehydrated tissues decreased in the same direction.

(c) Appearance of rehydration gradients (Figure 3I) so often observed may be explained by this reasoning (see below).

All frozen-dried muscle tissues obtained in our experiments not only rehydrated well, but also substantially restored their normal structural organization in detail. In fact, a rehydrated lyophilized sample may not be readily distinguishable from normal raw muscle tissue (compare Figures 3D and 3E). Moreover, this was true of rehydrated lyophilized muscle tissues pre-frozen at any of the three temperatures used (Figures 3B and 3G). The distinctness of cross-striations of the reconstituted muscle fibers in Figure 3B is particularly noteworthy. Therefore, as pointed out previously *(14, 15)*, cross-striations can be used as a criterion of the degree of reconstitution.

Some characteristic differences in these rehydrated tissues were noted. For tissues pre-frozen at either —80° C. or —17° C., partial interfibral

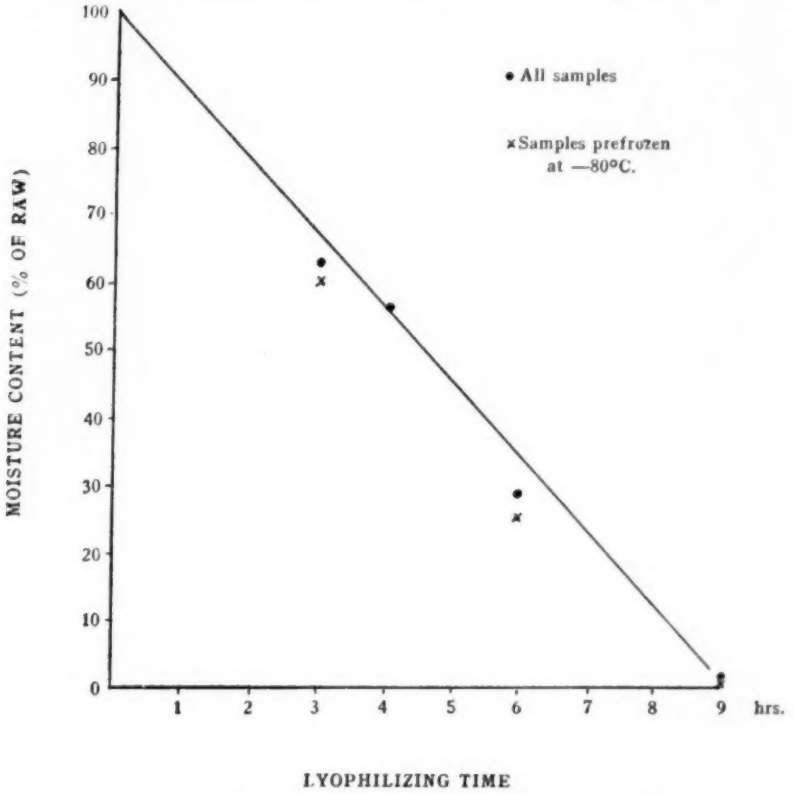
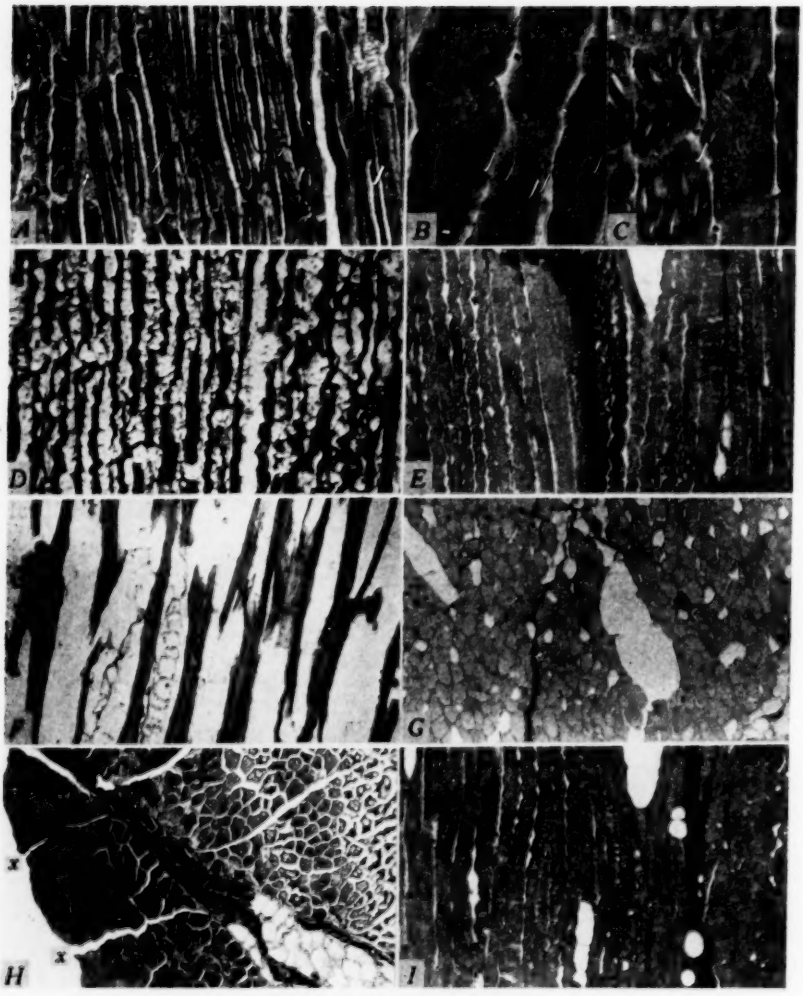


Figure 2. Rate of moisture removal by lyophilization of beef muscles



Description of Figure 3

Photomicrographs of 10 μ longitudinal sections (except G and H, transverse sections) of Biceps femoris stained with hematoxylin and Van Gieson's. × 215 for B and C; × 50 for the rest. A, D, F, and H were frozen-dried and immersed in absolute alcohol as the first step in processing;; B, C, E, G, and I, rehydrated lyophilized tissues, fixed in 10% neutral formalin.

A. Lyophilized tissue, pre-frozen at —150°C.
B. Pre-frozen at —150°C. lyophilized and rehydrated showing well rehydrated and reconstituted muscle fibers.
C. Pre-frozen at —150°C., lyophilized and rehydrated showing retention of ice spaces in the muscle fibers.
D. Lyophilized tissue pre-frozen at —80°C.
E. Rehydrated from tissues similar to D showing high degree of reconstitution.
F. Lyophilized tissue pre-frozen at —17°C.
G. Rehydrated from tissues similar to F showing good reconstitution.
H. Pre-frozen at —150°C. showing freezing gradients.
I. Portion of a rehydrated lyophilized sample pre-frozen at —80°C. to show rehydration gradients. (See text for details.)

space retention was quite common (see triangular space at top of Figure 3E and two oval spaces in Figure 3G). On the other hand, interfibral spaces in samples pre-frozen at —150°C. frequently failed to disappear completely (Figure 3C), especially in those fibers located near the center of the sample. As a result, such a rehydrated tissue would appear less reconstituted than those pre-frozen at the other two temperatures.

Furthermore, a frozen-dried slice of beef may not always reconstitute uniformly during rehydration. The structures of a rehydrated tissue, such as shown in Figure 3I, showed rehydration gradients which apparently were the result of decreasing water availability from the periphery (surface) to the center of the tissue (respectively, left and right of Figure 3I). The gradients were visible in (a) diameter of muscle fibers; (b) extent of structural reconstitution; and (c) return of endomysial spaces. All these decreased along the direction of water penetration.

HISTOCHEMICAL DETERMINATIONS

Sections of dehydrated and rehydrated muscle tissue were subjected to Carere-Comes staining technique for potassium. With low temperatures of pre-freezing, frozen-dried muscle tissue presented the same pattern in potassium distribution as fresh raw muscle tissue. Upon rehydration of frozen material, distribution of this mineral was nearly normal. A certain amount of displacement was noted which probably was due to lack of rapid pre-freezing and movement of water during rehydration.

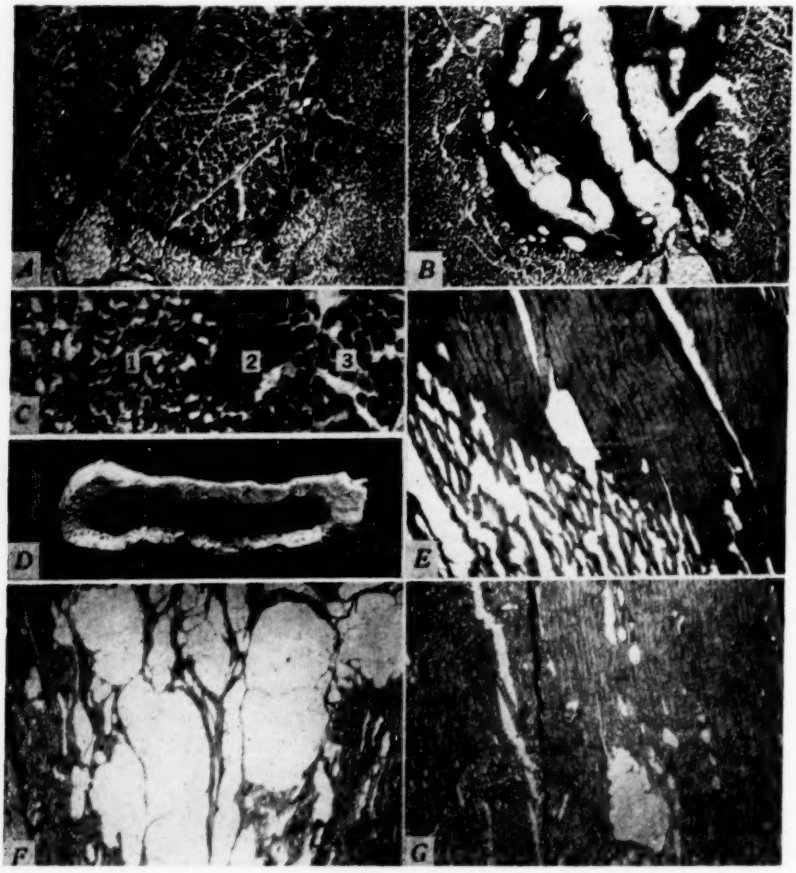
There was a qualitative difference in potassium distribution in samples pre-frozen at different temperatures. The least movement was found in tissues pre-frozen at —150°C. and the most in samples pre-frozen at —17°C. This naturally indicates *in situ* freezing at the lower temperature, while at higher temperature, freezing is less rapid. This would account for the movement of solutes and the formation of intercellular ice crystals.

Moisture data of samples pre-frozen at the 3 temperatures indicate that displacement of potassium probably is not a primary factor in the reconstitution or rehydration of muscle tissue, but that the relationship of space to fiber in the tissue is the important factor.

B. **Characteristics of beef muscle tissue after partial freeze-drying and final vacuum oven drying.** As indicated by the data (Table 1), complete dehydration of our samples required a minimum of 9-10 hours of lyophilization. A fair percentage of the samples were found to contain a centrally located core of varying size (Figures 4A and 4D). Such a core was composed essentially of raw muscle tissue, and contained all the residual moisture in the sample. Thus, the size of the core served as an index for the degree of dehydration in a sample or vice versa.

Moisture removal rate by lyophilization obtained from these samples was significantly faster than in samples of similar size dehydrated in warm-air cabinets or vacuum oven at 65° or 70°C. *(14)*. Furthermore, whereas the rate under the conventional methods was rapid at first, and dropped sharply to a sustained plateau (see Figure 1, *14*), that revealed by the lyophilized samples was uniform throughout since it was a nearly straight-line function of time (Figure 2).

From the unique structural makeup of a partially lyophilized beef sample, such as portrayed by Figures 4A and 4D, one may deduce that



Description of Figure 4

A. Transverse section through center of beef Biceps femoris muscle lyophilized for 6 hours showing raw core surrounded by frozen-dried muscle tissue. × 15

B. Sample in 4A after it was completely dehydrated in a vacuum oven at 45°C. Transverse section. × 15

C. An enlarged portion of 4A showing 3 distinct zones marking the boundary between the periphery and core, from left to right: Frozen-dried (1); transitional (2); and raw muscle fibers (3). × 50

D. Transverse section of a partially lyophilized Biceps femoris sample to show its core. × 3.2

E. Longitudinal section of Biceps femoris, incompletely lyophilized and followed by vacuum oven-drying at 45°C. showing the intact boundary between the periphery and core. × 44

F. Longitudinal section of Biceps femoris, incompletely lyophilized and followed by vacuum oven-drying at 70°C. to show its dehydrated core marked with enormous interfibral spaces. × 15

G. The same sample in 4F after it was rehydrated showing a fine state of reconstitution. × 15

sublimation proceeds from the sample's entire free (exposed) surface centrally, layer by layer, until the last trace of water farthest removed from the surface is removed. At any given moment during lyophilization the sample would consist of two distinct tissues, completely dried periphery and raw core. The relative size of the two evidently is a function of lyophilizing time at any given temperature and pressure in the lyophilizer *(6, 7, 8, 9)*. The rate of dehydration from such a tissue would be uniformly high because of an uninterrupted free passage of water vapor through the porous, spongy periphery. In contrast to this, one may recall that in samples subjected to warm-air dehydration, water passage was blocked as the tissue surface hardened and lost all its endomysial spaces *(4, 14)*.

Complete data on samples which were partially lyophilized are presented in Table 2. Most of these samples were subsequently dehydrated completely in a warm-air oven. Figure 4A illustrates the microscopic structure of a partially lyophilized sample. Its core was typically centrally located (the photograph was taken from one end of the core), and was surrounded completely by frozen-dried tissue without detectable gradients. The sharply defined core consisted of raw muscle tissue with normal endomysial spaces and muscle fibers of undiminished diameter (Table 2). However, there existed a narrow transitional zone (Figures 4A and 4C) which clearly marked the boundary between the periphery and the core. The muscle fibers in this zone were close together with little endomysial spaces between, indicating that moisture removal had just begun. Other indirect evidences that the residual moisture in such a sample was confined to the core were obtained: (a) muscle fiber diameter throughout the frozen-dried periphery was reduced to equal extent; (b) no difference was noted in muscle fiber diameter after varying periods of lyophilization nor did fiber diameter vary in relation to relative size of the periphery and core (Table 2); and (c) even after subsequent oven-drying, fiber diameter of the frozen-dried tissue remained unchanged (Table 2).

The results of further drying incompletely frozen-dried muscle tissues in a vacuum oven have revealed several points of interest (Table 2). The structure and fiber diameter of the periphery were not affected by the treatment. The core of the raw tissue behaved much as expected—muscle fibers underwent a 30% reduction in diameter and all endomysial spaces disappeared, but there were neither signs of merging of adjacent fibers nor decreased visibility of cross-striations. Most notably, an enormous system of spaces interpolating the dried muscle fibers had emerged (Figure 4B). Conceivably, these spaces came into existence by replacing the endomysial spaces. Volume-wise, the new spaces would be equal to the original endomysial spaces plus those created through the shrinkage of the dehydrated muscle fibers. Moisture-wise, the core lost most of its moisture (Table 2).

The oven-dried partially lyophilized samples rehydrated to a surprisingly high degree, only slightly below the level obtained by the completely frozen-dried samples considered in section A. Both the structural picture and muscle fiber diameter indicated far better reconstitution than was ever possible with samples dried without the freeze-drying treatment. On the average, these samples returned to a moisture content and muscle fiber diameter equal to, respectively, 65-92% and 60-91% of the raw tissue

(Table 2). The state of structural reconstitution was well portrayed by Figure 4G (contrast it with Figure 4F before rehydration). The reconstitution changes consisted of (a) great, though by no means complete, reduction of the enormous interfibral spaces; (b) a fair degree of return of endomysial spaces; and (c) substantial recovery of fiber diameter together with good cross-striations. Merging of muscle fibers was not observed. This high reconstitution was generally true of oven-dried cores in partially lyophilized samples regardless of oven temperature or dehydration time. Great consistency of the data in these respects is especially noteworthy.

Core-containing samples were sectioned and stained for potassium localization. Samples dried at 45°C. exhibited a movement of potassium in the core area typical of high temperature dehydration. All rehydrated samples indicated some movement of this ion. As explained previously, it was once thought that this ion might influence the subsequent degree of rehydration and reconstitution. In that respect, it is interesting to note that frozen-dried, vacuum oven-dried (at 45°C.) cores of samples exhibited a histology of dehydrated tissue similar to samples dried at 70°C. However, after rehydration, the former samples regained 80% of the fiber diameter, whereas the latter samples regained only 65% of the control. In both cases, the potassium was similar qualitatively, yet the 45°C. dehydrated core rehydrated quite well. This again indicates that potassium distribution is probably not necessarily a factor in rehydration or reconstitution.

That pre-freezing had nothing to do with the rehydration of the dried tissue was evidenced by the fact that samples pre-frozen at—80°C. and dehydrated in a vacuum oven at 45°C. without lyophilization rehydrated poorly. Other experiments were conducted as follows: some incompletely frozen-dried samples were dried in the vacuum oven *in toto* after which their periphery was stripped off and the core alone was rehydrated. In a second group of samples the core was isolated after lyophilization, vacuum oven-dried without periphery, and rehydrated. In a third group of samples the raw core was removed and replaced by a cylinder of raw meat. These were then vacuum oven-dried and the cores rehydrated. The performance of the cores in these three groups of samples is presented in Table 3. The results show that, on the basis of muscle fiber diameter recovery, the core surrounded by a lyophilized periphery rehydrated best, next were the bare cores, and lastly the cores which were placed within the lyophilized peripheries as raw cylinders. Thus, the presence of a peripheral, porous, lyophilized tissue appears to have contributed toward the increased rehydratability of the core. Moisture-wise, however, the data indicated that the bare cores rehydrated to a slightly higher level than those protected by a periphery. But there is complete agreement that the raw unlyophilized cores rehydrated least of all.

Some explanation is needed for this high core rehydratability in partially lyophilized muscle samples. First of all, we must assume as we did before, that the core had not been subjected to extensive chemical changes throughout the treatment. Secondly, since the continuous boundary between the periphery and the core (Figure 4A) remained intact during oven-drying (Figures 4B, 4E, and 4F), while the muscle fibers in the core shrank as they lost moisture, a space system was bound to emerge. The same significance may be attributed to these spaces as to those in com-

TABLE 3

Moisture content and muscle fiber diameter of unlyophilized raw core, and of raw core in lyophilized Biceps femoris samples followed by vacuum oven-drying at 45° C. for 3 hours

Sample No.	Treatment	Moisture content (%)			Muscle fiber diameter (μ)		
		Raw	After dehydration*	After rehydration	Raw	After dehydration	After rehydration
1	Core isolated after lyophilization and dried in vacuum oven alone.	73.4	36.0	67.3	40.1	26.7	31.1
2		73.4	23.1	61.9	40.1	26.7	36.2
3		73.4	39.6	67.5	40.1	36.1	36.2
4	Entire lyophilized sample dried in vacuum oven, but core alone was rehydrated.	73.4	0.6	61.2	40.1	22.4	40.4
5		73.4	0.6	61.5	40.1	28.4	38.3
6		73.4	1.2	64.3	40.1	24.8	40.7
8	A raw core was inserted into a lyophilized sample, dried in vacuum oven and the core was rehydrated.	73.4	54.2	63.0	40.1	26.2	38.3
9		73.4	3.2	46.4	40.1	26.3	28.7
10		73.4	0	38.0	40.1	24.0	30.0

*Assuming that the residual water in an incompletely lyophilized sample is in the core.

pletely frozen-dried samples insofar as their being instrumental to rehydration of the tissue is concerned. Judging from past experiments using warm-air drying techniques *(11)*, we are of the opinion that the periphery (frozen-dried tissue) acts in some fashion to reduce any serious temperature effect on the core. But exactly what the role is, we do not know at present.

The results from core-containing samples have given promise to possibilities of actually shortening the costly lyophilization process by further drying of the partly frozen-dried products at temperatures as high as 70°C. This would mean, of course, a considerable saving on production cost especially in view of the fact that the resulting oven-dried products were not markedly inferior to or different from completely frozen-dried beef samples. Only one reservation should be made, however, i.e., these findings were obtained from experimental-size samples and strictly under the conditions the experiments were carried out in our laboratory.

SUMMARY

Slices of *Biceps femoris* and *Semimembranosus* muscles were pre-frozen at −150°C., −80°C., or −17°C. and lyophilized under pressures of 0.001 to 0.3 mm. mercury. Samples so dried by sublimation revealed a rate of moisture removal that was uniform and significantly faster than in similar samples dehydrated by warm-air methods. Partially lyophilized samples contained a central core of raw muscle tissue. Freeze-drying proceeded from the surface.

The morphological pattern of frozen-dried muscle tissue depended upon pre-freezing rate which was determined by pre-freezing temperature. Despite differences in structural organization of lyophilized tissues, pre-frozen at three different temperatures, all had these common characteristics:

(a) no change in total volume of tissue;

(b) no sign of denaturation of muscle fiber protein; and

(c) presence of a space system which thoroughly interpolated dried muscle fibers.

All frozen-dried samples, regardless of pre-freezing temperature, rehydrated to 85-90% of original moisture content, and their muscle fibers returned to 89-98% of their original diameter.

Potassium distribution of frozen-dried muscle tissue was essentially that of fresh raw muscle. A limited degree of potassium displacement was found on rehydration.

Partially lyophilized core-containing samples were subsequently dried in a vacuum oven at 23°, 45°, or 70°C. until complete or nearly complete tissue dehydration was obtained. Oven-drying did not change the peripheral sublimated tissue; sample volume was also unchanged; and the original boundary between periphery and core remained intact. Water loss from the core brought simultaneous muscle fiber shrinkage and emergence of an enormous space system. These changes were not accompanied by muscle fiber merging or cross-striation loss.

Oven-dried partially lyophilized samples rehydrated to a significantly higher level than was achieved by dehydration at 65° or 70°C. without freeze-drying. The tissue achieved a fair degree of reconstitution.

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A HISTOLOGICAL AND HISTOCHEMICAL STUDY OF BEEF DEHYDRATION. V. SOME FACTORS INFLUENCING THE REHYDRATION LEVEL OF FROZEN-DRIED MUSCLE TISSUE[a]

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Having studied the morphological aspects of frozen-dried muscle tissue (*1*), we were interested in learning what factors could possibly influence the level of rehydration. Muscle fiber orientation in relation to absorbing surfaces, sample thickness, rehydrating fluid temperature, pH, osmotic pressure, and vacuum rehydration were considered in this phase of the investigation.

MATERIALS AND METHODS

Six choice carcasses, approximately 8 days after slaughter, were utilized. Samples were taken from the *Biceps femoris*, *Semitendinosus*, and *Semimembranosus* muscles of the round. These samples were either strips, ranging in size from ¼ x ⅜ x 2½ inches to 1 x ⅜ x 2½ inches in length, weighing from 5 to 25 g., or cylinders ranging from ½ to 1 inch in diameter by 2½ inches in length, weighing from 5 to 23 g.

Samples were pre-frozen at −80°C. for 30 minutes and frozen-dried from 5½ to 23 hours in a laboratory lyophilizer. Vacuum during freeze-drying ranged from .008 to 0.4 mm. mercury. Ground samples of raw meat were dried in a vacuum oven for 18 hours at 65°C. to determine the moisture content.

Some samples were rehydrated in demineralized water for varying lengths of time at 22°C. and at 55°C. In another experiment, the osmotic pressure of the rehydrating solution was varied by adding sodium chloride to demineralized water in the following concentrations: 0.3%, 0.9%, and 1.5%. At the same time, pH was adjusted to approximately 4.0, 7.0, and 10.0 with NaOH or HCl. Other samples were rehydrated under vacuum. Samples were held under vacuum for 5 minutes prior to admitting water into the vacuum chamber. In all rehydration experiments, samples were blotted carefully prior to weighing.

EXPERIMENTAL RESULTS AND DISCUSSION

Effect of muscle fiber orientation. To test orientation as a factor in rehydration, samples were cut so that muscle fibers were either perpendicular (transverse samples) or parallel (longitudinal samples) to the largest surface areas. When ¼ inch strips of *Semimembranosus* were completely or nearly completely lyophilized, transverse strips dehydrated to a lower moisture level in 5½ hours than longitudinal strips (Table 1). Similar effects from muscle fiber orientation were noted for cylinders ½ inch in diameter but not for 1 inch diameter cylinders of tissue.

Both transverse strips and transverse cylinders of lyophilized beef muscle tissue rehydrated more rapidly and to a higher level than did corresponding samples with muscle fibers parallel to the surface with the greatest area (Table 1 and Figure 1).

The degree and rate of rehydration apparently depend upon the directness and shortness of the water route into the tissue. There was less structural interference

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TABLE 1

Comparative moisture content of frozen-dried muscle strips and cylinders with different muscle fiber orientation after rehydration for increasing lengths of time

Type of sample	No. of samples	% of residual moisture average	Average moisture content and standard deviation after rehydration					
			3 min. %	10 min. %	15 min. %	30 min. %	1 hr. %	3 hr. %
Transverse strips	10	0.4	67.3 ± 2.0	68.0 ± 3.0	67.8 ± 1.9	....	....	....
Longitudinal strips	10	4.7	55.8 ± 6.4	58.0 ± 4.2	58.6 ± 5.4	59.8 ± 3.3	....	....
Transverse cylinders, ½ inch	5	0	58.4 ± 2.1	60.0 ± 2.6	....	62.3 ± 2.2	....	....
Longitudinal cylinders, ½ inch	5	0	44.5 ± 5.3	47.4 ± 4.5	....	51.7 ± 4.5	....	....
Transverse cylinders, 1 inch	3	1.6	....	48.5 ± 2.8	....	51.3 ± 7.0	53.7 ± 1.0	56.8 ± 0.4
Longitudinal cylinders, 1 inch	2	7.3	....	38.6 ± 2.7	....	42.3 ± 0.1	44.8 ± 0	48.8 ± 2.0

Moisture content of raw = 73.3%.

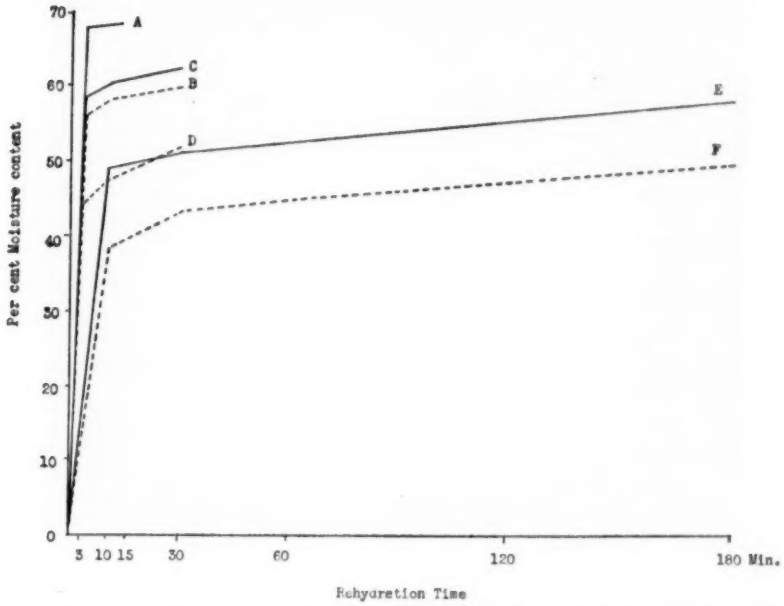

Figure 1. Comparative rate of rehydration and rehydration levels of lyophilized beef muscles with transverse (curves A, C, E) and longitudinal (curves B, D, F) orientation of fibers in both strips (A and B) and cylinders (C, D, E, F) based on data in Tables 1 and 2.

where the muscle fibers were perpendicular to the greatest area of the tissue. The same principle held for moisture removal from tissue *(2)*.

To illustrate structurally, we have chosen two of the ½-inch cylinders representing the cross-sectional view of a transverse cylinder and its longitudinal counterpart. A thin slice of the cylinder containing longitudinally arranged muscle fibers (Figure 2) exhibited only surface rehydration. The central light area was unrehydrated completely; only a narrow rim at the periphery, much darker in shade, was rehydrated tissue. On the other hand, the corresponding section from the cylinder with fibers running perpendicular to the cylinder's long axis (Figure 3) indicated a thorough penetration of water.

The results presented above appear to be conclusive that for any given thickness the transverse samples rehydrated faster as well as to a higher level than the longitudinal samples.

Effect of thickness of samples. To test the influence of thickness of a sample on the rate and level of rehydration, ½-inch and 1-inch cylinders of *Semitendinosus* were compared. The former weighed an average of 5 g., while the 1-inch samples weighed approximately five times as much. After 21 hours of lyophilization, we noted that the ½-inch samples were dehydrated completely while the 1-inch samples retained some moisture (Table 1).

For the transverse and longitudinal sectional cylinders, respectively, the ½-inch group rehydrated much faster and to a higher level at the end of 3 minutes (Table 1). The 1-inch cylinders did not attain this level even at the end of 3 hours in the demineralized water. These data show conclusively that rehydration rate and level are influenced by the thickness of the sample. At the same time, the data also indicate that for

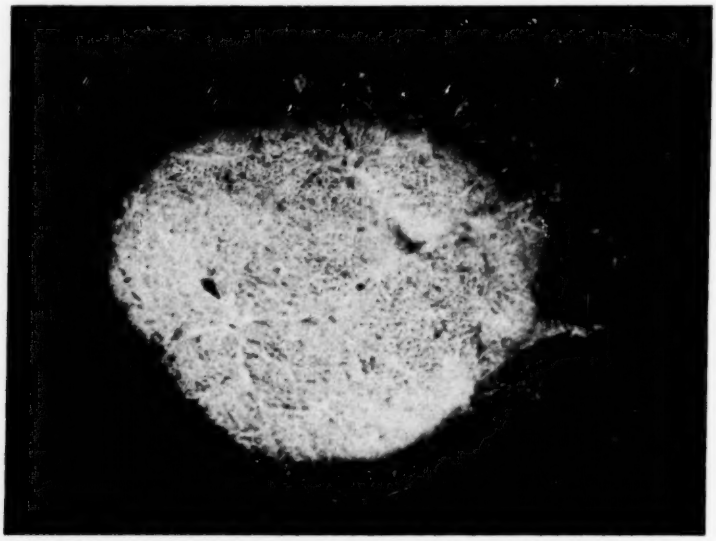

Figure 2. Cross section view of a rehydrated ½-inch Semitendinosus cylinder with muscle fibers parallel to axis of cylinder. × 9

Figure 3. Longitudinal section view of rehydrated ½-inch Semitendinosus cylinder with muscle fiber oriented perpendicular to axis of cylinder. × 9

small samples (5 g.) muscle fiber orientation is more important than sample thickness as a factor influencing rehydration. This was shown by the fact that ½-inch transverse cylinders rehydrated faster and to a higher level than did the ½ inch longitudinal strips. However, this relationship holds only within certain limits of sample thickness. Probably with increasing thickness of a sample the significance of muscle fiber orientation continually decreases.

Effect of temperature of the rehydrating substrate. When ½ inch diameter cylinders of lyophilized *Semimembranosus* muscle with transverse fiber orientation were rehydrated in water, no effect of temperature of the water was found (Table 2). At the

TABLE 2

Comparative moisture content of completely frozen-dried Semimembranosus samples rehydrated at room temperature (22°C.) and at 55°C.

Temperature of rehydration	Number of samples	% average moisture and standard deviation after rehydration			
		3 min.	20 min.	1 hr.	3 hr.
22°C.	10	49.7 ± 6.0	53.3 ± 5.7	56.6 ± 5.7	61.6 ± 5.3
55°C.	10	49.2 ± 2.6	54.6 ± 1.0	59.1 ± 2.7	60.4 ± 1.7

temperatures tested, 22°C. and 55°C., the temperature of the rehydrating water did not influence the rate or extent of rehydration of the frozen-dried muscle tissue.

Effect of pH and osmotic pressure of rehydration solutions. In another experiment, completely or nearly completely lyophilized, transverse sections of *Semimembranosus* (½ x ⅞ x 2½ inches) were rehydrated in demineralized water whose pH and osmotic pressure were varied. The data (Table 3) indicate that the level of rehydration decreased as the rehydrating solution became more hypertonic. The highest level of rehydration was observed in demineralized water.

Another factor which appears to influence rehydration is pH. In all cases, regardless of the salt concentration, the highest level of rehydration occurred at approximately pH 7.

Effect of vacuum rehydration. This experiment was devised to test the rehydration level of frozen-dried samples of muscle tissue rehydrated under vacuum. The conventional method used for rehydration was immersion of the sample in demineralized water for 30 minutes.

TABLE 3

Comparative moisture content of completely lyophilized Semimembranosus samples rehydrated at different salt concentrations and pH's

% salt concentration	pH	% Moisture Content			
		5 min.	10 min.	15 min.	30 min.
0.3	4.3	52.4	54.5	56.2[1]	58.3[1]
	7.1	62.8	63.8	64.5	65.9
	10.5	54.4	56.1	60.6[1]	61.9[1]
0.9	3.9	41.8	43.4	48.3[1]	50.6[1]
	5.8	49.8	52.8	54.5	57.0
	10.0	47.6	49.4	49.0	51.1[1]
1.5	3.9	46.2	48.4	57.4[1]	59.2[1]
	7.3	59.3	60.3	61.0	62.3
	10.2	46.0	48.5	54.9[1]	56.9[1]
Control, deionized water	3.6	58.3	59.9	65.3[1]	66.9[1]
	7.7	61.4	62.9	63.2	64.7
	9.9	57.1	58.7	64.2[1]	65.6[1]

[1]Average of two samples.

As the data indicate (Table 4), the samples under vacuum rehydrated more rapidly and to a higher level than samples rehydrated without vacuum. It was noted that all of the samples that were vacuum rehydrated were of dark red-brown color. This probably was due to the fact that the myoglobin was in the reduced state *(4)*.

TABLE 4

Comparative moisture content of completely lyophilized Semimembranosus samples rehydrated under vacuum and by conventional method

Sample No.		% Moisture Content after Rehydration		
		5 min.	10 min.	15 min.
1	A	56.3	58.0	59.7
	B	58.0		
2	A	53.2	55.3	55.5
	B	60.2		
3	A	56.7	58.6	58.9
	B	63.2		
4	A	56.2	58.5	59.7
	B	58.3		
5	A	61.0	62.4	62.6
	B	66.2		
6	A	62.1	65.0	65.5
	B	65.7		
7	A	52.0	54.8	56.3
	B	65.7		
8	A	44.8	46.9	47.8
	B	63.4		

A = Conventional method.
B = Under vacuum.

SUMMARY

Six factors believed to influence rehydration of frozen-dried muscle tissue have been investigated: (a) orientation of muscle fibers with reference to main absorptive surfaces of the sample; (b) thickness of the sample; (c) temperature of rehydrating solution; (d) osmotic pressure of rehydrating solution; (e) pH of rehydrating solution; and (f) rehydration under vacuum.

For samples ranging in weight from 5 to 25 g., and in strips or cylinders up to 1 inch in thickness, rehydration was significantly faster and to a higher level in transverse than in longitudinal samples.

The rate and level of rehydration decreased as thickness of sample increased.

No appreciable difference in either rate or level of rehydration was found between similarly treated frozen-dried samples rehydrated in demineralized water at 22°C. and 55°C.

Increasing hypertonicity of the rehydrating solution by addition of sodium chloride adversely affected the rehydration level. Best results were obtained with demineralized water alone.

The highest level of rehydration occurred in solutions in which the pH was near 7.0, regardless of the osmostic pressure.

Samples of frozen-dried muscle tissue under vacuum rehydrated faster and to a higher level than samples rehydrated without vacuum.

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NUTRITIONAL SUPPLEMENTATION OF LACTIC ACID FLORA OF SAUERKRAUT[a, b]

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In commercial fermentation of sauerkraut it is considered that the higher the rate of fermentation and the faster the rate of production of acid, especially in the first stages of the fermentation period, the smaller are the risks of spoilage.

The purpose of this work was to test the effect of certain additives, such as vitamins, amino acids, and salts, and of bacterial starters on the rate of fermentation, final pH, final total acidity, and quality of sauerkraut.

EXPERIMENTAL METHODS

The cabbage used for this work was purchased locally. It was produced in Florida and in South Georgia and shipped to this area fresh and sound.

The cabbage was shredded by machine into ¼-inch shreds, weighed, and packed in containers in alternate layers with salt (sodium chloride). Water was added to all containers since the production of juice by plasmolysis was too slow in the small containers used. The cabbage juice in some cases failed to cover all the cabbage in each container. Because of the small total amount of cabbage, growth of spoilage microorganisms on the surface of the exposed cabbage influenced all the contents of the jar. For this reason in all experiments a mixture of 67.02% cabbage, 30.71% water, and 2.26% salt was used. Waxed wood slats were used to keep the cabbage below the surface of the liquid. The fermentation was conducted at room temperature. The mean temperature for Experiment I was 15.5°C.(60°F.), for Experiment II it was 23.9°C. (75°F.), and for Experiment III it was 23.9°C.(90°F.).

Experiment I consisted of 8 treatments replicated 4 times. The constituents composing each treatment and the concentration used are listed in Table 1.

Experiment II consisted of 3 treatments replicated 4 times. The first treatment consisted of a control containing cabbage, water, and salt. The second treatment consisted of the control plus additives, and the third consisted of the control, additives, and carbon dioxide bubbled from the bottom of the container through the packed cabbage for 30 minutes. The additives and the concentrations at which they were used may be found in Table 2.

Experiment III consisted of 20 treatments replicated 3 times. The design of this experiment appears in Table 3. A list of the additives that were used and their concentration is presented in Table 4.

Pure culture inocula were used in Experiment III. The bacteria used in this experiment were obtained directly from the American Type Culture Collection, Georgetown University Medical School, Washington, D. C. The following microorganisms were used: *Lactobacillus brevis* No. 4006 (A.T.C.C.), *Lactobacillus plantarum* No. 4008 (A.T.C.C.), and *Leuconostoc mesenteroides* No. 8042 (A.T.C.C.). Stab cultures were incubated at 37.0°C.(98.6°F.) in micro assay culture agar *(1)* for 48 hours and subsequently kept in storage at 2.2°C.(36.0°F.). The cultures were carried in triplicate. One of the cultures

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[b]A part of the thesis presented by Mr. Mark G. Schwartz to the Graduate Faculty of the University of Georgia in partial fulfillment for the degree, Master of Science in Agriculture, 1953.

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TABLE 1

Design and composition of Experiment I

Treatment	Ingredients	Concentration in total mixture (per 100 ml.)
1	Control	
	Two-gallon containers:	
	cabbage 9.50 lbs.	
	water 4.40 lbs.	
	salt 0.31 lbs.	
	Five-gallon containers:	
	cabbage 24.00 lbs.	
	water 11.00 lbs.	
	salt 0.81 lbs.	
2	Control plus	
	dextrose	0.50 g.
3	Control plus	
	sodium acetate	0.10 g.
	magnesium sulfate	0.04 g.
	calcium phosphate	0.05 g.
	ammonium phosphate	0.05 g.
4	Control plus	
	dextrose	0.50 g.
	sodium acetate	0.10 g.
	magnesium sulfate	0.04 g.
	calcium phosphate	0.05 g.
	ammonium phosphate	0.05 g.
5	Control plus	
	biotin	0.5 μg.
6	Control plus	
	dextrose	0.50 g.
	biotin	0.05 μg.
7	Control plus	
	thiamine	20 μg.
	riboflavin	40 μg.
	niacin	10 μg.
	pyridoxine	20 μg.
	pantothenic acid	30 μg.
	folic acid	0.5 μg.
	biotin	0.5 μg.
8	Control plus	
	dextrose	0.50 g.
	thiamine	20 μg.
	riboflavin	40 μg.
	niacin	10 μg.
	pyridoxine	20 μg.
	pantothenic acid	30 μg.
	folic acid	0.5 μg.
	biotin	0.5 μg.

was used exclusively for the inoculation of new culture tubes every 7 days. The other two were used for the preparation of the inocula for the treatments.

The inocula for the treatments were prepared as follows: The bacteria were transferred to tubes of micro assay culture agar and incubated for 48 hours. At the end of the incubation period the cultures were transferred to micro inoculum broth *(1)*. After

an additional 48 hours this broth culture was added to approximately 150 ml. of micro assay culture agar contained in two-liter Erlenmeyer flasks and incubated for 24 hours at 37.0°C.(98.6°F.). An abundant growth was obtained. The growth was washed from the surface of the agar with 100 ml. of the solution to be used in the respective treatments and then 20-ml. portions were added to each jar in which inocula were to be used.

Added nutrients. The nutrients which were added to the kraut were chosen on the basis of previous research which had demonstrated that these substances are essential for the growth of lactic-acid-producing bacteria in pure cultures *(4, 11, 12, 13, 15, 16, 17, 18)*.

TABLE 2

Additives of Experiment II

Additives	Concentration in total mixture (per 100 ml.)
Dextrose	0.5 g.
Sodium acetate	0.1 g.
Magnesium sulfate	0.04 g.
Calcium phosphate	0.05 g.
Ammonium phosphate	0.05 g.
Potassium mono-hydrogen phosphate	0.12 g.
Potassium di-hydrogen phosphate	0.12 g.
Ferric chloride	0.0001 g.
Zinc sulfate	0.00176 g.
Magnesium sulfate	0.004 g.
Thiamine	20 μg.
Riboflavin	40 μg.
Niacin	10 μg.
Pyridoxine	20 μg.
Pantothenic acid	30 μg.
Folic acid	0.5 μg.
Biotin	0.5 μg.
p-Aminobenzoic acid	200 μg.
Inositol	500 μg.
Tryptophane	0.008 g.
l-Cystine	0.02 g.
Cysteine	0.02 g.
Asparagine	0.02 g.
Adenine sulfate	0.002 g.
Guanine hydrochloride	0.002 g.
Uracil	0.002 g.
Xanthine	0.002 g.
Tween No. 80[1]	0.002 g.
Filtered tomato juice	1 g.

[1]Tween 80 (polyoxyethylene sorbitan monoleate) supplied by Atlas Powder Co., Wilmington, Delaware.

Criteria used to evaluate the rate of fermentation. The criteria used to evaluate the rates of fermentation were total acidity and hydrogen-ion concentration. Aliquots were taken of the liquid of the kraut, well below the surface, after 0, 1, 2, 4, 6, 9, 12, 15, 21, 24, 27, and 30 days. At these intervals pH measurements were made in a Beckman pH meter, Model G, and converted to hydrogen-ion concentration in grams per liter. A known volume of the liquid was titrated with a standard alkali and the total acidity calculated as percentage of lactic acid.

RESULTS

The total acidity increased in two stages. The rate of fermentation slowed down after 18, 21, and 12 days for Experiments I, II, and III, respectively. The rate of increase of total acidity per day to the point of which the rate of fermentation slowed down was calculated by the regression coefficient *(19)*. Analysis of variance *(14)* was

TABLE 3

Design of Experiment III

Treatment
1. Control (cabbage 9.50 lbs., water 4.40 lbs., and salt 0.36 lbs.)
2. Control + *Lactobacillus brevis*
3. Control + *L. plantarum*
4. Control + *Leuconostoc mesenteroides*
5. Control + *L. brevis*, *L. plantarum*, and *L. mesenteroides*
6. Control + additives[1]
7. Control + additives[1] and *L. brevis*
8. Control + additives[1] and *L. plantarum*
9. Control + additives[1] and *L. mesenteroides*
10. Control + additives[1] and *L. brevis*, *L. plantarum*, and *L. mesenteroides*
11. Control + carbon dioxide[2]
12. Control + carbon dioxide[2] and *L. brevis*
13. Control + carbon dioxide[2] and *L. plantarum*
14. Control + carbon dioxide[2] and *L. mesenteroides*
15. Control + carbon dioxide[2] and *L. brevis*, *L. plantarum*, and *L. mesenteroides*
16. Control + carbon dioxide[2] + additives[1]
17. Control + carbon dioxide[2] + additives[1] and *L. brevis*
18. Control + carbon dioxide[2] + additives[1] and *L. plantarum*
19. Control + carbon dioxide[2] + additives[1] and *L. mesenteroides*
20. Control + carbon dioxide[2] + additives[1] and *L. brevis*, *L. plantarum*, and *L. mesenteroides*

[1]The list of additives is presented in Table 4.
[2]The carbon dioxide was bubbled through the packed cabbage for two hours.

TABLE 4

Additives of Experiment III

Additives	Concentration in total mixture (per 100 ml.)
Dextrose	5 g.
Proteose peptone	1 g.
Sodium acetate	2.5 g.
Ammonium chloride	0.6 g.
Potassium di-hydrogen phosphate	0.12 g.
Potassium mono-hydrogen phosphate	0.12 g.
Magnesium sulfate	0.040 g.
Manganese sulfate	0.003 g.
Ferrous sulfate	0.002 g.
Thiamine	100 μg.
Pyridoxine	400 μg.
Pyridoxamine	70 μg.
Pyridoxal	300 μg.
Pantothenic acid	100 μg.
Riboflavin	100 μg.
Niacin	200 μg.
Biotin	0.5 μg.
Folic acid	2 μg.
Inositol	500 μg.
p-Aminobenzoic acid	200 μg.
Adenine sulfate	0.002 g.
Guanine hydrochloride	0.002 g.
Uracil	0.002 g.
Xanthine	0.002 g.
Tween No. 80[1]	0.2 g.

[1]Tween 80 (polyoxyethylene sorbitan monoleate) supplied by Atlas Powder Co., Wilmington, Delaware.

used to compare these values statistically. The same statistical design was employed to evaluate acidity and hydrogen-ion concentration data obtained on the thirtieth day of fermentation.

Experiment I. All rates of increase were higher than the control at the 5.0% level of probability. The rates of increase of treatments No. 4 (sodium acetate, inorganic salts, and dextrose) and No. 8 (vitamin combination and dextrose) were higher than the control at the 1% level of probability. Figure 1 shows the mean rates of increase and the final total acidities of several treatments of Experiment I.

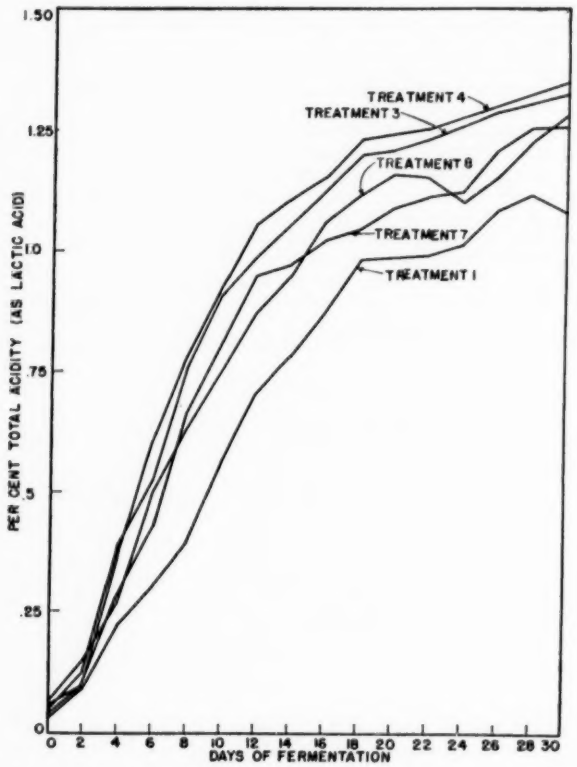

Figure 1. Increase in total acidity of treatment No. 1 (control) and of Nos. 3, 4, 7, and 8 of Experiment I.

The final total acidities, expressed as percentage of lactic acid, of treatments in Experiment I are shown in Table 5. With the exception of treatment No. 6 (dextrose and biotin) the resulting total acidities of the finished kraut were significantly higher than the control. The final total acidities of treatments No. 3 (sodium acetate and inorganic salts), No. 4 (sodium acetate, inorganic salts and dextrose), No. 5 (biotin), No. 7 (vitamin combination), and No. 8 (vitamin combination and dextrose), were higher than that of the control. The differences were highly significant.

The final hydrogen-ion concentrations in grams per liter, for Experiment I, are summarized in Table 5. The only hydrogen-ion concentration significantly higher than the control was found in treatment No. 8 (dextrose and vitamin combination).

Experiment II. The values for the final total acidity for Experiment II are summarized in Table 6. Both treatments No. 2 (additives) and No. 3 (additives and carbon dioxide) showed higher total acidity values than the control. The difference was significant at the 1.0% level of probability. Figure 2 shows the mean rates of increase and the final total acidities.

The final hydrogen-ion concentrations for the treatments of Experiment II showed no statistically significant differences among treatments or replications.

Experiment III. A highly significant increase in the daily rate of acid production was observed in treatments to which all the nutrients listed in Table 4 had been added.

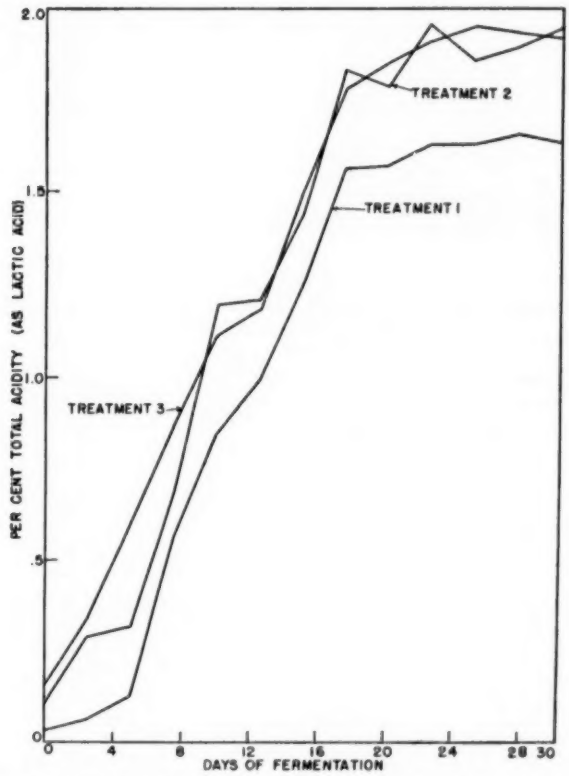

Figure 2. Increase in total acidity of treatment No. 1 (control), and of Nos. 2 and 3 of Experiment II.

The rate of acid production for samples inoculated with *Leuconostoc mesenteroides* was shown to be significantly lower than the control. Statistical analysis failed to demonstrate any beneficial effect from added carbon dioxide. Figure 3 shows the mean rates of increase and the final total acidities.

The final total acidity was significantly higher at the 1.0% probability level for treatments to which all the nutrients listed in Table 4 had been added. No other statistical differences were found in the data obtained from the total acidity determinations in this experiment. Carbon dioxide and pure and mixed culture inocula did not affect significantly the final total acidity. The data may be found in Table 7.

TABLE 5

Percentage total acidity, expressed as grams of lactic acid per 100 ml., and hydrogen-ion concentration, after 30 days of fermentation, for Experiment I

Treatments	Replications								Mean	
	1		2		3		4			
	Total acidity	$[H^+]$[1]	Total acidity	$[H^+]$[1]	Total acidity	$[H^+]$[1]	Total acidity	$[H^+]$[1]	Total acidity	$[H^+]$[1]
	(%)	(g./lt.)	(%)	(g./lt.)	(%)	(g./lt.)	(%)	(g./lt.)	(%)	(g./lt.)
1	0.07	3.98	1.04	3.63	1.08	3.71	1.21	3.23	1.08	3.64
2	1.41	5.01	1.13	4.26	1.12	3.38	1.04	3.16	1.18	3.95
3	1.35	2.09	1.28	1.95	1.34	3.89	1.34	3.63	1.33	2.89
4	1.36	2.04	1.30	1.95	1.38	4.46	1.32	4.46	1.34	3.23
5	1.30	4.67	1.24	4.89	1.11	4.16	1.21	4.26	1.21	4.49
6	1.07	3.89	1.13	4.07	1.22	4.57	1.20	4.89	1.15	4.35
7	1.24	3.98	1.29	3.98	1.24	4.16	1.30	4.26	1.27	4.10
8	1.35	5.01	1.20	5.12	1.29	4.26	1.25	4.46	1.27	4.71
Mean	1.26	3.83	1.20	3.73	1.22	4.07	1.23	4.04	1.23	3.92

[1] Each figure is multiplied by 10,000.
Total acidity: LSD for treatment at the 5% level: 0.09.
Total acidity: LSD for treatment at the 1% level: 0.13.
Hydrogen-ion concentration: LSD for treatment at the 5% level: 0.86.

TABLE 6

Percentage total acidity, expressed as grams of lactic acid per 100 ml., after 30 days of fermentation, Experiment II

Treatments	Replications			Mean
	1	2	3	
1	1.63	1.70	1.57	1.63
2	1.88	1.92	2.01	1.93
3	1.92	1.92	1.90	1.91
Mean	1.81	1.85	1.83	1.83

LSD for treatment at the .05% level = 0.17.
LSD for treatment at the .01% level = 0.26.

TABLE 7

Percentage total acidity, expressed as grams of lactic acid per 100 ml., and hydrogen-ion concentration, after 30 days of fermentation, for Experiment III[1]

Inocula	Without carbon dioxide				With carbon dioxide				Means of inocula treatments	
	No additives		With additives		No additives		With additives			
	Total acidity	$[H^+]$[2]	Total acidity	$[H^+]$[2]	Total acidity	$[H^+]$[2]	Total acidity	$[H^+]$[2]	Total acidity	$[H^+]$[2]
	(%)	(g./lt.)	(%)	(g./lt.)	(%)	(g./lt.)	(%)	(g./lt.)	(%)	(g./lt.)
None	1.66	4.21	3.18	2.04	1.63	4.10	3.02	2.54	2.38	3.22
L. brevis	1.70	4.07	3.17	2.01	1.72	4.18	2.85	2.10	2.36	3.09
L. plantarum	1.68	4.11	2.90	2.06	1.67	4.01	2.96	2.43	2.38	3.24
L. mesenteroides	1.65	4.14	2.65	1.63	1.63	4.28	2.49	1.94	2.11	3.00
Mixed cultures	1.77	4.04	2.69	1.66	1.79	4.28	2.59	1.82	2.21	2.95
Means	1.69	4.11	2.98	1.88	1.69	4.24	2.78	2.16	....	....

[1] Each value is the mean of 3 replications.
[2] Each figure is multiplied by 10,000.
Total acidity: LSD for additives at the 5% level: 0.79.
Total acidity: LSD for additives at the 1% level: 1.05.
Hydrogen-ion concentration: LSD for additives at the 5% level: 0.72.
Hydrogen-ion concentration: LSD for additives at the 1% level: 0.96.

The hydrogen-ion values may be also found in Table 7. The addition of nutrients caused a reduction in the final hydrogen-ion concentration. This reduction was statistically significant at the 1.0% level of probability. The use of inocula and of carbon dioxide had no statistically significant effect on the final hydrogen-ion concentration.

In sauerkraut fermentation, it is important that the pH decrease rapidly during the first few days of fermentation so that organisms other than the aciduric lactic-acid-producing organisms will be inhibited. On that account, the influence of the various experimental variables was studied to ascertain whether any treatment resulted in a more rapid decrease in pH than the controls. For this purpose, the number of days which it took for the pH of each treatment to decrease to pH 4.0 was determined. The mean value

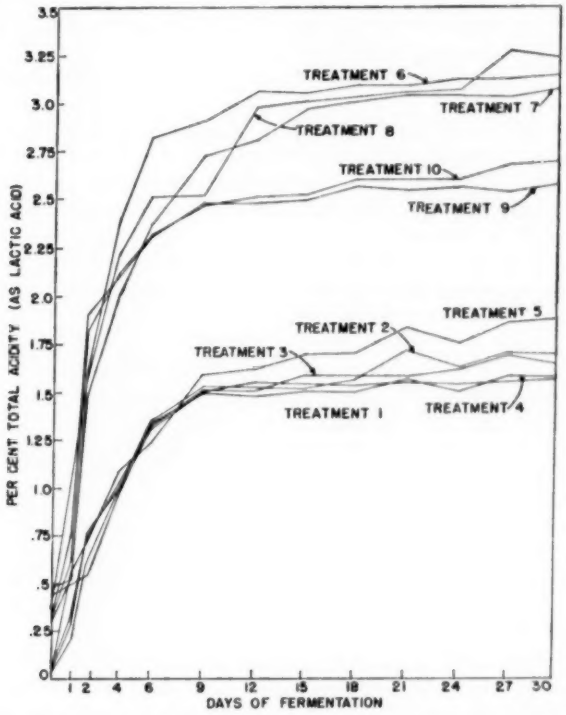

Figure 3. Increase in total acidity of treatment No. 1 (control), and Nos. 2, 3, 4, 5, 6, 7, 8, 9, and 10 of Experiment III.

for the 3 replications was calculated. It was found that the treatments that included starters of one, two, and three species of bacteria and no additives decreased in pH in general at a faster rate than the controls. The treatments that contained the additives and no bacterial starters, and the treatments that contained the additives and bacterial starters decreased in pH at a slower rate than the controls. All the mean values for the controls and for the treatments containing bacterial starters and no additives were lower than 3.0. These results do not show that the addition of CO_2 influenced the decrease of pH to 4.0. However, in experiments involving pickles, to be reported later, the addition of CO_2 did decrease the pH significantly faster than that of controls during the first days of the fermenting period.

DISCUSSION AND CONCLUSIONS

The hydrogen-ion concentration, expressed in grams per liter, was used to make comparisons in preference to the pH values because pH values do not lend themselves as readily to statistical calculations.

Although the temperature was not controlled in this study, the rate of fermentation appeared to be accelerated when the prevailing temperature was high. This may be shown by the length of time required for the rate of increase in total acidity of the different experiments to level off. The total acidity of the sauerkraut in the experiment initiated in the summer required only 12 days to level off, while in experiments run in cooler seasons of the year the rate of increase in total acidity broke at 18 and 21 days. This is in agreement with the work of Parmele *et al. (5)*, Pederson *(9)*, and of Pederson and Albury *(10)*, who found a close correlation between temperature and rate of fermentation. In contrast to results reported by Pederson *(8)*, kraut was fermented at temperatures of 30°C.(86°F.) and above without spoilage. Experiment III showed a highly significant difference in rate of fermentation and in final total acidity values between treatments containing additives (dextrose, mineral salts, vitamins, amino acids, etc.) and the control.

Results obtained in Experiment I agree with the work of Fellers *et al. (3)* who demonstrated that the final acid content of sauerkraut could be increased by the addition of dextrose.

The controls in Experiments II and III were somewhat slower in reaching the same total acidity values than some of the commercial batches reported by Pederson *(9)*, fermented at approximately the same temperature. The final total acidities of treatments containing additives were always above 2% and often above 3%. Pederson *(8)*, reported that fermentation is complete when the total acidity is between 1.5 and 2%. Although direct comparisons of the total acidity and pH of sauerkraut diluted with brine and then fermented cannot be made with commercial batches in which brine is not used, during the course of the work reported here it was observed that the total acidity and the pH of the batches with additives consistently showed a faster rate of fermentation, higher total acidity, and lower pH than commercial batches.

The work of Snell *et al. (10)*, showed that the addition of sodium acetate accelerated the growth of lactic acid bacteria in pure culture and also had a buffering effect on the media. Experiment I showed the same effects in the fermentation of sauerkraut. A buffering action of sodium acetate was also demonstrated in the lactic acid fermentation of cucumber pickles by Durrence *(2)*.

Carbon dioxide was added to produce microaerophilic conditions in the sauerkraut during the first stages of fermentation. When carbon dioxide was used, hydrogen-ion concentration and total acidity were higher during the first 2 days of fermentation. However, after 2 days, no difference in the hydrogen-ion concentration and total acidity was observed which could have been attributed to added carbon dioxide. The results of this study have not shown any other significant effect of added carbon dioxide upon the fermentation of sauerkraut.

Except for a pure culture of *Leuconostoc mesenteroides*, the use of pure or mixed culture inocula of *Lactobacillus brevis, L. plantarum,* and *Leuconostoc mesenteroides* had no effect on the final total acidity, on the final hydrogen-ion concentration, or on the rate of fermentation. These results disagree with those obtained by Pederson *(6)* who found that sauerkraut inoculated with the mixed cultures of lactic-acid-producing bacteria completed fermentation earlier than the controls, and that the use of cultures of *Leuconostoc mesenteroides* starters produced a kraut of low total acidity.

In Experiment III a brownish discoloration was observed in the treatments containing additives as compared with the controls. The texture of the samples containing the additives was somewhat less firm than the controls. No off-flavors were observed, with the exception of higher acidity in the finished sauerkraut, which was of a degree that would not be considered objectionable. Off-odors of slight intensity were observed in the samples containing additives. These odors somewhat lowered the quality of the product.

SUMMARY

The effect of certain added nutrients and bacterial starters upon the fermentation of sauerkraut was studied.

Dextrose, sodium acetate, a mixture of inorganic salts and the vitamin combination increased the final total acidity of sauerkraut, whether used alone or in combinations, with the exception of the combination of dextrose and biotin.

Use of pure or mixed starter cultures of *Lactobacillus brevis, L. plantarum,* and *Leuconostoc mesenteroides* did not produce a significant increase in the final hydrogen-ion concentration, in the final total acidity, or in the rate of fermentation. However, at the beginning of the fermentation period, the pH of the samples treated with pure starter cultures decreased to a value of 4.0 at a faster rate than the samples to which no starter cultures were added.

Addition of carbon dioxide did not increase the hydrogen-ion concentration or the total acidity, except for the first two days of the fermentation period.

Some off-odors and off-colors were observed in sauerkraut fermented in the "complete" medium; the flavor was nevertheless normal except for higher acidity.

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